Calmodulin from the eucestoda Hymenolepis diminuta: an investigative study

Eastlake, Jane Louise

Award date:
1994

Awarding institution:
University of Bath

CALMODULIN FROM THE EUCESTODA

Hymenolepis diminuta:

AN INVESTIGATIVE STUDY.

Submitted by **Jane Louise Eastlake** *nee Brook*

For the degree of Doctor of Philosophy

of the University of Bath

1994.

J L Eastlake

ABSTRACT

An investigation to establish the importance of the calcium binding protein, calmodulin in the rat tapeworm Hymenolepis diminuta was undertaken.

Initially an attempt was made to isolate the gene encoding for calmodulin. It was found that there is probably one gene copy in Hymenolepis diminuta. However no sequence data could be obtained so that comparison with other calmodulin genes was not possible.

A method to isolate calmodulin from the cestode was developed using pig thymus tissue. Four methods were tried however it was found that the most suitable method for isolating calmodulin from pig thymus was not the same as that for cestode. The isolated calmodulin was analysed by SDS-PAGE where it had a molecular weight of 17kD and showed a migratory shift in the presence and absence of calcium. Its biological activity was assessed using a phosphodiesterase assay system. But it was discovered that phosphodiesterase is an unstable enzyme so that the identity of the isolated protein was further verified using ELISA and Western blot.

Furthermore the calmodulin binding proteins were isolated and analysed by SDS-PAGE. These appeared to have molecular weights comparable with known calmodulin binding proteins.

The distribution of calmodulin within the cestode was investigated using immunocytochemical techniques using both fluorescent and transmission electron microscopy. Calmodulin was discovered associated with cellular structures to which it has previously been found in other eukaryotes.

Overall this study has provided further evidence that calmodulin probably has a central role in regulating cestode metabolism, comparable with that seen in other eukaryotic organisms.

ACKNOWLEDGEMENTS

My thanks to my supervisors Dr. W.J.D. Whish and Dr. C. Branford-White for their combined support and enthusiasm throughout this project. I am also especially grateful to Dr. Whish for the scientific discussions and technical advice that were always freely given.

I am also very grateful to the following people who have given me the following technical assistance: Dr. A. Rogers, immunochemistry; Dr. P. Harris, molecular biology; Mrs. U. Potter, electron microscopy and Mr. N. Dewes-Broughton, photographic development.

My thanks also to my colleagues in the laboratory and the biochemistry department who have provided continual support and friendship, in particular Joan Whish, Marjan Kasrean, Ross Philips, Steffi Kappus and Ali Yildrum. It has been a pleasure working alongside them.

Very special thanks to my husband, David Eastlake, who has been a stalwart of support throughout my PhD. Furthermore, he has also provided computing and printing facilities together with invaluable assistance in the preparation of poster and paper presentations, not to mention this thesis.

Finally I am very grateful to the Science and Research Engineering Council for their financial support of this project, without which it would not have been possible to study such a fascinating organism.

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LIST OF ABBREVIATIONS

ADP .... adenosine diphosphate

AMP ...... adenosine monophosphate

ATP .... adenosine triphosphate

ATPase ..... adenosine triphosphatase

CaM .... calmodulin

CaM-BP .... calmodulin binding protein

cAMP ...... cyclic adenosine monophosphate

cDNA ...... complementary DNA

CoA .... acetyl coenzyme A

CTAB ..... cetyl trimethyl ammonium bromide

dATP ...... deoxyadenosine triphosphate

dCTP ...... deoxycytidine triphosphate

DEPC ...... diethyl pyrocarbonate

dGTP ...... deoxyguanosine triphosphate

DNA ...... deoxyribonucleic acid

DNAase .... deoxyribonuclease

dNTP ...... deoxynucleotide triphosphate

dTTP ...... deoxythymidine triphosphate

EDTA: ..... ethylene diamine tetraacetic acid

EGTA ..... ethylene-glycol-bis-β-aminoethyl-ether-N,N,N,N,-tetraacetic acid.

EF Hand: ... a structural motif found at calcium binding sites in some proteins consisting of a helix-loop-helix, named EF after the E- and F- helices of parvalbumin.

ELISA ..... enzyme linked immunosorbant assay

FITC ...... fluoroscein isothiocyanate

HEPES ..... N-2-hydroxyethyl-piperazine-N-2-ethanesulphonic acid

IPTG ...... isopropyl-β-D-galactoside

kcal .... kilo calories

MAPS ..... microtubule associated proteins

MEM ...... minimal essential medium

MLCK ..... myosin light chain kinase

MOPS ..... 3'-N-morpholiopropane sulphonic acid

M_r .... molecular weight

mRNA ..... messenger ribonucleic acid

NAD ...... nicotinamide adenine dinucleotide (oxidized form)

NADH ..... nicotinamide adenine dinucleotide (reduced form)

NADP ..... nicotinamide adenine dinucleotide phosphate (oxidized form)

NADPH .... nicotinamide adenine dinucleotide phosphate (reduced form)

NBL .... Northern Biological Laboratories

OD .... optical density

PBS .... phosphate buffered saline

PCR .... polymerase chain reaction

PDE .... phosphodiesterase

P_i .... inorganic orthophosphate

PP_i .... inorganic pyrophosphate

PMSF ...... phenylmethylsulphonylfluoride

RNA ...... ribonucleic acid

RNAase ......ribonuclease

rRNA ....ribosomal RNA

SDS ....sodium dodecyl sulphate

SDS-PAGE ...sodium dodecyl sulphate polyacrylamide gel electrophoresis

SSC ....NaCl/sodium citrate/SDS

STOPS ....stable tubule only polypeptides

TBE ....tris/EDTA/borate

TBS ....tris buffered saline

TCA ....trichloroacetic acid

TE ....tris/EDTA

TEMED ......N,N,N,N-tetramethylethylenediamine

TEN9 ....tris/EDTA/NaCl

TFP ....trifluoroperazine

TLC ....thin layer chromatography

TMB ....tetramethylbenzidine

Tris ....tris(hydroxymethyl)methylamine

tRNA ....transfer RNA

TWEEN ......polyoxethylene sorbitan monolaurate

UDP ....uridine diphosphate

UDP-glu ......uridine diphosphate glucose

UMP ....uridine monophosphate

X-gal 5-bromo-4-chloro-3-indoyl-β-galactoside

1 INTRODUCTION

1.1 CALMODULIN

Calmodulin is a ubiquitous calcium binding protein found in both prokaryotic and eukaryotic cells (Ruben 1983). It is a member of a family of structurally related calcium binding proteins that include troponin C, parvalbumin and myosin light chain (Klee & Vanaman 1983). All of these proteins share a common helix-loop-helix structural motif at the calcium binding sites that are referred to as the EF-hand, after the E- and F- helices of parvalbumin (Kretsinger 1976).

Calmodulin consists of 148-150 amino acid residues that are arranged to form a tertiary dumbbell like structure. See Figure 1 and Table 1. Around 50 of these amino acid residues are aspartate and glutamate, which accounts for the low isoelectric point of calmodulin, pI=4.3. There is a low proline, histidine and tyrosine content whilst there are no cysteines or tryptophan residues. The result of which is a very flexible molecule capable of withstanding low pH, extremes of temperature and 8M urea. Calmodulin also displays a high phenylalanine to tyrosine ratio, with 8 phenylalanine to only 2 tyrosine residues, 1 in plants (Wallace, Tallant & Cheung 1980; Cheung 1982).

Only two post-translational modifications have been found in calmodulin. The first is a trimethylated lysine at position 115, and the second is acylation of the N-terminus. See Figure 2. The structural significance of the trimethylated lysine is still unknown however the acylation of the N-terminus is protective and may form an anchor for insertion into plasma membranes (Klee, Crouch & Richman 1980).

The tertiary structure of calmodulin consists of two globular lobes connected by a

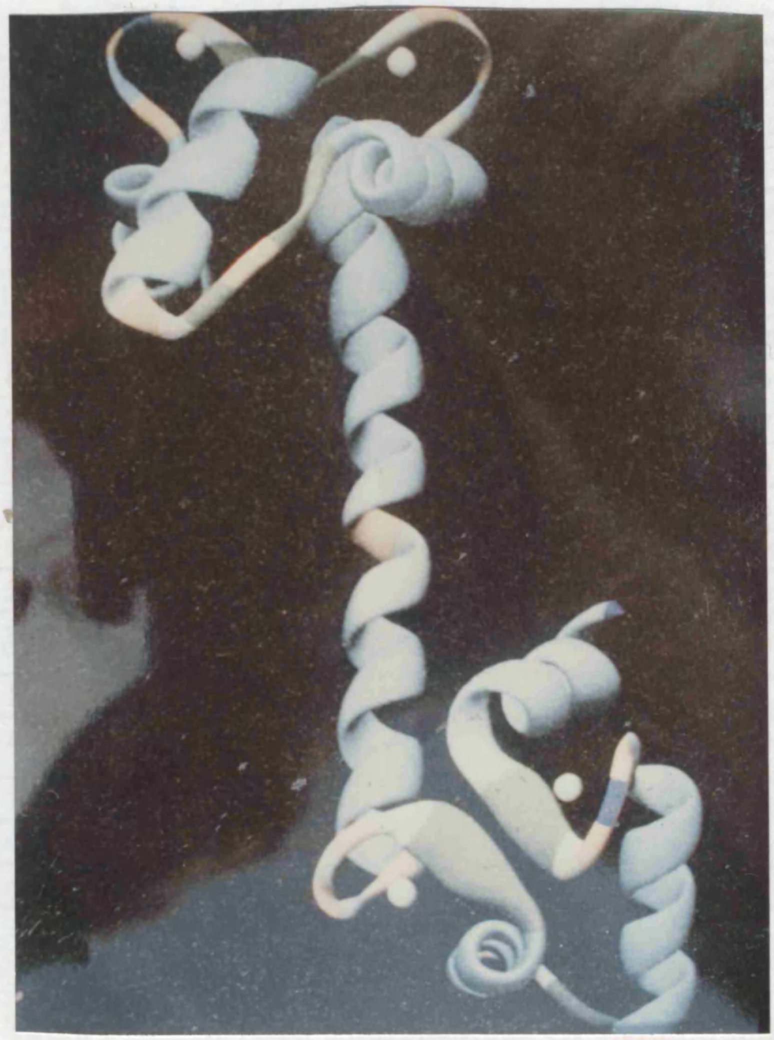


Figure 1. Structure of Calmodulin

This shows the x-ray structure of rat testes calmodulin. It shows two globular domains that contain the calcium binding sites, which are connected by a seven turn α-helix. This figure is reprinted from "Biochemistry" by D.Voet and J.Voet, copyright ©1990 John Wiley & Sons, Inc.. Reprinted by permission of John Wiley & Sons, Inc., and is based on data supplied by M.Carlson. The x-ray structure was determined by C.Bugg.

source / amino acid	Bovine brain (residues/ molecule)	Renilla reniformis (residues/ molecule)	Metridium senile (residues/ molecule)	Tetrahyme-nia pyriformis (residues/ molecule)	Spinach leaf (residues/ molecule)	Barley leaf (residues/ molecule)	Chlamydo-monas reinhardtii (moles/ 16700g)	dictyo-stelium discio-deum (residues/ molecule)	Arabacia punc-tulata (moles/ 16700g
aspartate	23	23	23	23	25	27.8	22.4	24	23
threonine	12	12	12	11	9	8.9	9.8	10	10.4
serine	4	5	5	4	4	5.5	5.6	6	4.9
glutamate	27	25	26	25	25	24.6	27.2	26	25.4
proline	2	2	2	2	2	2.2	2.4	2	2.12
glycine	11	11	11	11	10	11.8	12.2	11	11.5
alanine	11	10	10	11	11	11.7	11.2	10	10.3
cysteine	0	0	0	0	1	-	-	0	0
valine	7	7	7	6	8	7.4	7	7	7.1
methionine	9	9	9	8	8	8.1	6.8	9	9.4
isoleucine	8	8	8	9	7	7	5.3	8	7.9
leucine	9	9	9	12	11	11.6	10	10	9.5
tryosine	2	1	1	1	1	1.3	1.4	2	1.9
phenyl-alanine	8	9	9	8	9	8.5	8.7	8	8.1
histidine	1	1	1	2	1	1.5	2.3	1	0.9
trimethyl-lysine	1	1	1	1	1	1.1	0	0	0.6
lysine	7	8	8	7	9	8	10	8	8.2
tryptophan	0	0	0	0	0	-	0	0	0
arginine	6	6	6	6	5	4.9	6.3	6	6.2

Table 1 Amino Acid Compositions Of Calmodulin

This shows the amino acid composition from different sources. Note that the numbers of each of the various amino acids is very constant (Burgess et al. 1983).

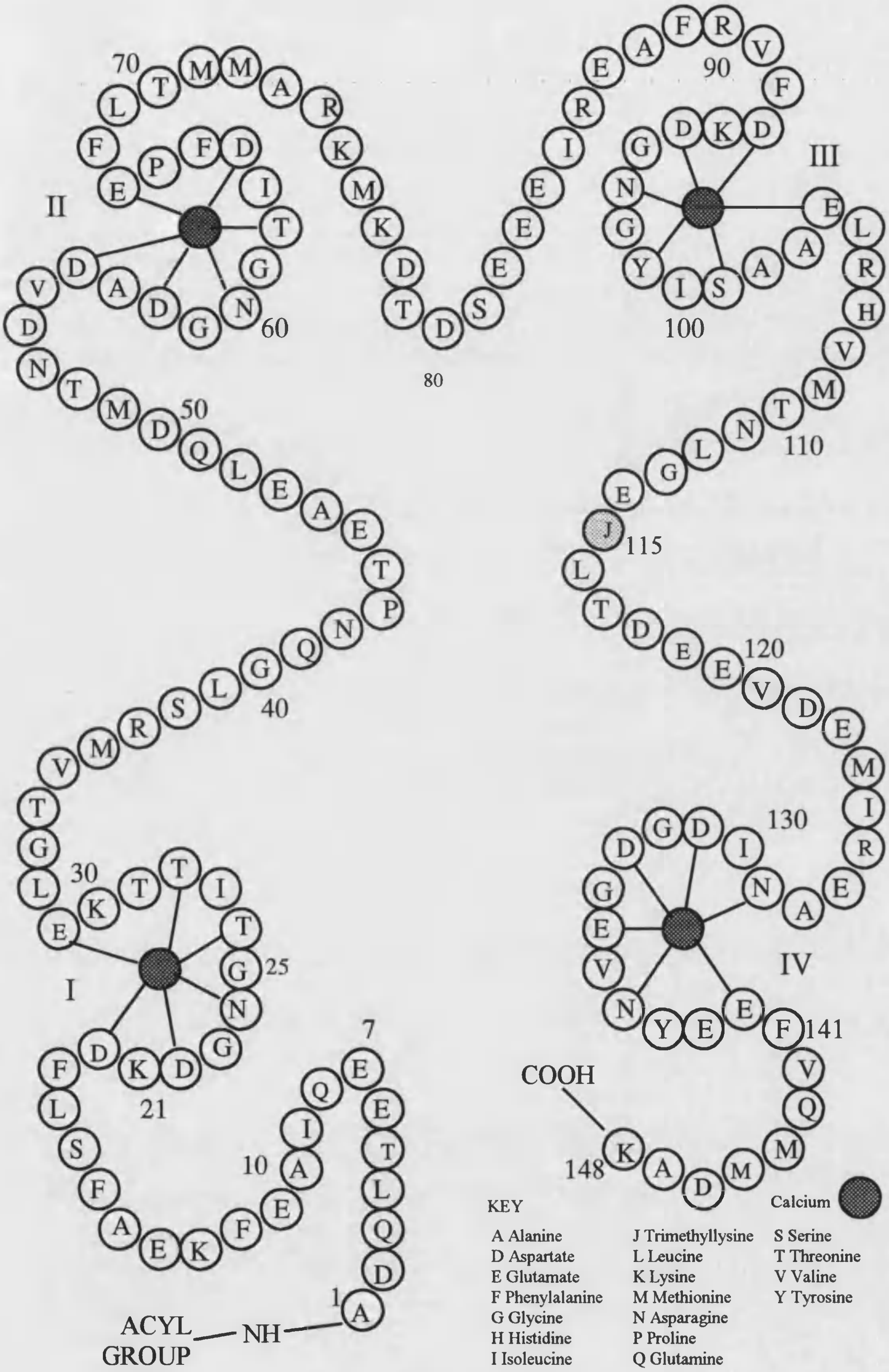


Figure 2. Arrangement Of Amino Acids Of Calmodulin

Diagram of the amino acid sequence for bovine brain calmodulin (Cheung 1982).

42Å central helix with an overall length of 65 Å (Ikura et al. 1992). The central helix is functionally very important as it possesses some degree of flexibility thereby enabling the calmodulin molecule to interact simultaneously with target domains and with calcium. Each lobe contains a pair of EF-hand loops, which is composed of a linear sequence of approximately 30 amino acids where two nearly perpendicular alpha helices flank a 12 residue calcium binding loop (Klee & Vanaman 1982; Babu et al. 1985).

In all eukaryotic calmodulin so far characterised, four calcium-binding sites have been identified with K_d values, for calcium, of $\approx 1\mu M$ (Klee, Crouch & Richman 1980; Klee & Vanaman 1982; Babu et al 1985; Salvato et al. 1986). Two of these are high affinity binding sites and two are low affinity as shown by ^{43}Ca NMR studies. Binding to each pair of sites appears to be positively co-operative. The two binding domains within the carboxyl terminal half of calmodulin, sites III and IV, are saturated before the amino terminal domains, sites I & II (Vogel 1988). However, the calcium binding sites have a lower affinity for calcium than some other substances such as EDTA and EGTA. The reason for this is that calcium binding proteins need to bind calcium reversibly, hence they bind calcium weakly (Bugg 1992). This allows for subtle variations in the calcium affinity thereby allowing biological responses to be initiated only at suitable concentrations of calcium which in the cell is 10^{-7} to 10^{-6}M (James 1992).

Upon binding calcium, calmodulin undergoes a large conformational change accompanied by a 5-10% increase in the α-helix content from the value of 28-45% found in a calcium free solution (Klee, Crouch & Richman 1980; Klee 1980). Concomitant with the binding of the calcium molecules is a large shift in the spectroscopic properties of calmodulin. Calmodulin has an unusual spectrum in that its main absorbance maxima is at 253nm rather than the 280nm peak found in many other proteins. This is because of the low phenylalanine to tryosine ration. A summary of calmodulins spectroscopic properties are shown in table 2 (Klevit 1983).

Due to the flexible nature of the protein it is very heat stable. It is an acid protein being soluble at pH7 and insoluble at pH4 (Klee, Vanaman 1983). In eukaryotic cell's calmodulin can represent up to 1% of total cellular protein and is generally found in the cytosol. On SDS-PAGE calmodulin without calcium, migrates with a molecular weight range of 13.5kD, in prokaryotes, to 17kD, in eukaryotes, however when calcium is present it migrates anomously with a molecular weight of 24kD.

1.1.1 CALMODULINS FUNCTION

Calmodulin regulates a large number of proteins, mainly enzymes, in response to changing cellular calcium levels (Yaswen 1990). This regulation relies on the fact that calmodulin specifically binds to calcium and not other cations. For example the cellular concentration of magnesium is between 1-5mM, and the level of potassium is 100mM, whilst the intracellular calcium levels vary only between 10^{-7}M and 10^{-6}M. It is clear that this specificity is due to the unique structure of calmodulin (Vogel 1988). However one might ask why is calcium the control cation.

Calcium has been selected by nature over many other cations as a messenger molecule. The reason for this selection is probably due to the chemical properties of calcium that enable it to be involved with both tight and specific interactions. As a divalent cation it has a higher affinity for polyanionic sites, on proteins, over monovalent ions such as sodium or potassium. Its ionic radius is 0.99 Å and enables it to fit into the pockets created on the surface of proteins, by the folding of

Property	Values
UV Absorbance	
Absorbance maxima	253,258,265, 268.5, 276 nm
Absorbance minima	250 nm
$\varepsilon^{1\%}/_{277nm}$	1.8-2.0 (vertebrates) 0.9-1.0 (invertebrates, plants)
$\varepsilon^{1\%}/_{253nm}$	0.95 (vertebrates)
Circular Dichroism	
Minima	207, 222 nm (far UV) 261, 268.5 nm (near UV)
$[\theta]_{222nm}$ [(deg.cm^2)dmol^{-1}] no calcium (1.2mM EGTA) calcium (0.3mM	 -11,500±500 -15,000±500
Optical Rotary Dispersion	
$[m']_{231nm}$ [(deg.cm)dmol^{-1}] no calcium calcium (0.1mM)	 -5700 -7500

Table 2. Spectroscopic Properties of Calmodulin

This shows a summary of the spectroscopic properties of calmodulin (Klevit 1983).

polypeptide backbones and the extension of amino acid side chains. It is able to interact with eight electron donors, usually oxygen atoms. By comparison, magnesium can only interact with six electron donors and has an ionic radius of 0.65Å. This means that magnesium would not completely fill the pockets created on proteins resulting in water molecules filling up the remaining space, which would greatly weaken the interaction of magnesium and the protein (Davis & Thorner 1986).

In response to increasing levels of cellular calcium, calmodulin molecules bind calcium. These calcium calmodulin complexes have no intrinsic activity but act by binding to other proteins. The action of binding calcium results in a conformational change in calmodulin, resulting in the exposure of hydrophobic residues that otherwise face the interior of the molecule. These can then interact with hydrophobic residues present on target proteins to effect binding (Jarret & Madhaven 1991). See Figure 3. It has been shown that in the presence of a calmodulin binding protein the binding of calcium to calmodulin is enhanced. This is because when camodulin has bound calcium to domains III and IV, surfaces are exposed which can then initiate the binding of calmodulin to the calmodulin-binding site of the target protein. Simultaneously a "message" is propagated through the central helix to the N-terminal half of calmodulin. The N-terminal calcium binding sites I and II undergo a conformational change that increases their affinity for calcium. Once the calcium has filled sites I and II, other hydrophobic domains are exposed which can increase the interaction with the target protein (Vogel 1988; Kretsinger 1992).

Many proteins that are regulated by calmodulin can also be activated by anionic lipids, which are thought to mimic the hydrophobic anionic sites on calmodulin. There are several models to account for the interaction of calmodulins with other

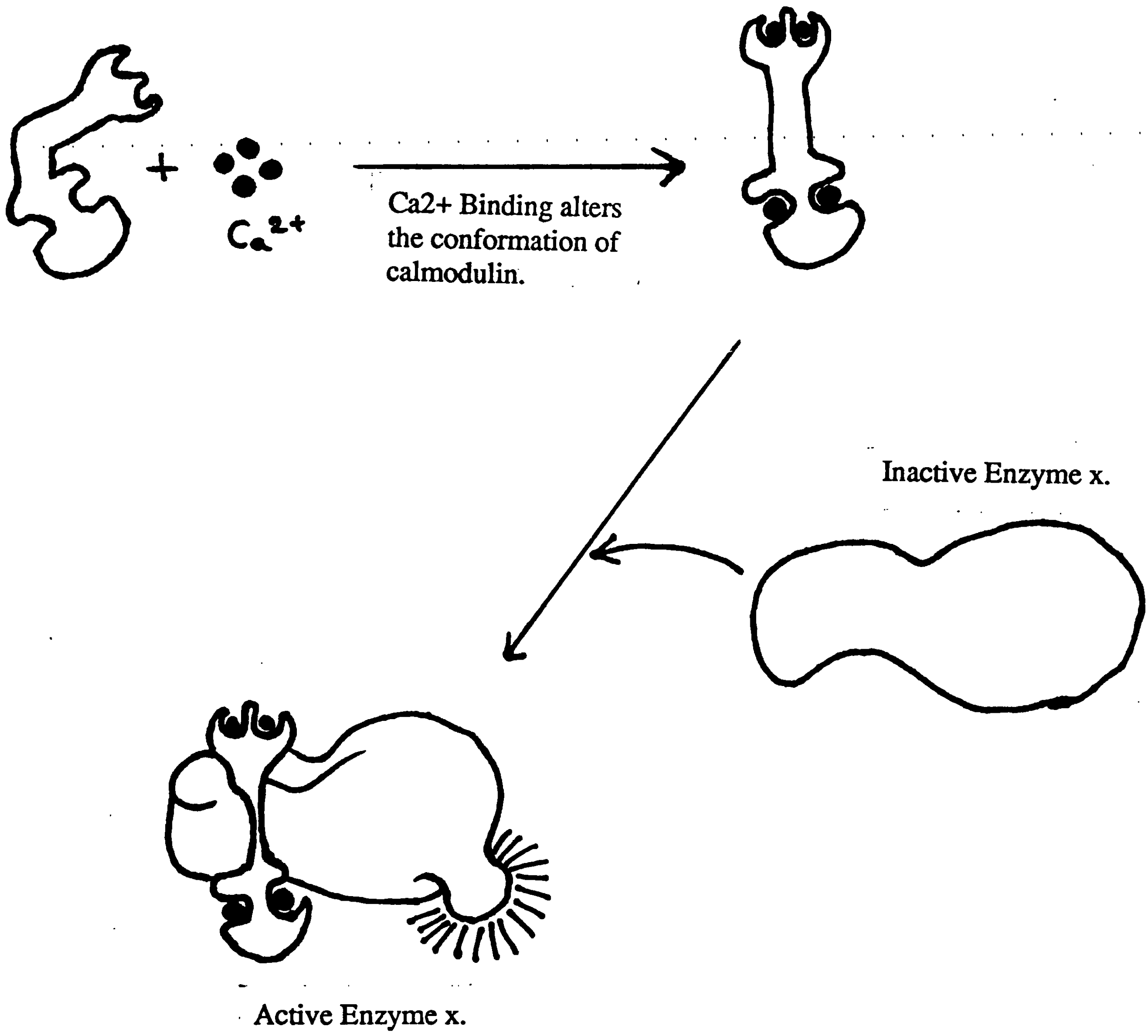


Figure 3. Mechanism Of Calmodulin Interaction With Target Proteins

Four calcium ions bind to calmodulin causing a conformational change, which enables calmodulin to interact with its target proteins. Reprinted with permission from "Molecular Biology Of The Cell" 2nd. Edition. Copyright ©1989 B.Alberts et al. Reprinted By Permission Of Garland Publishing Inc.

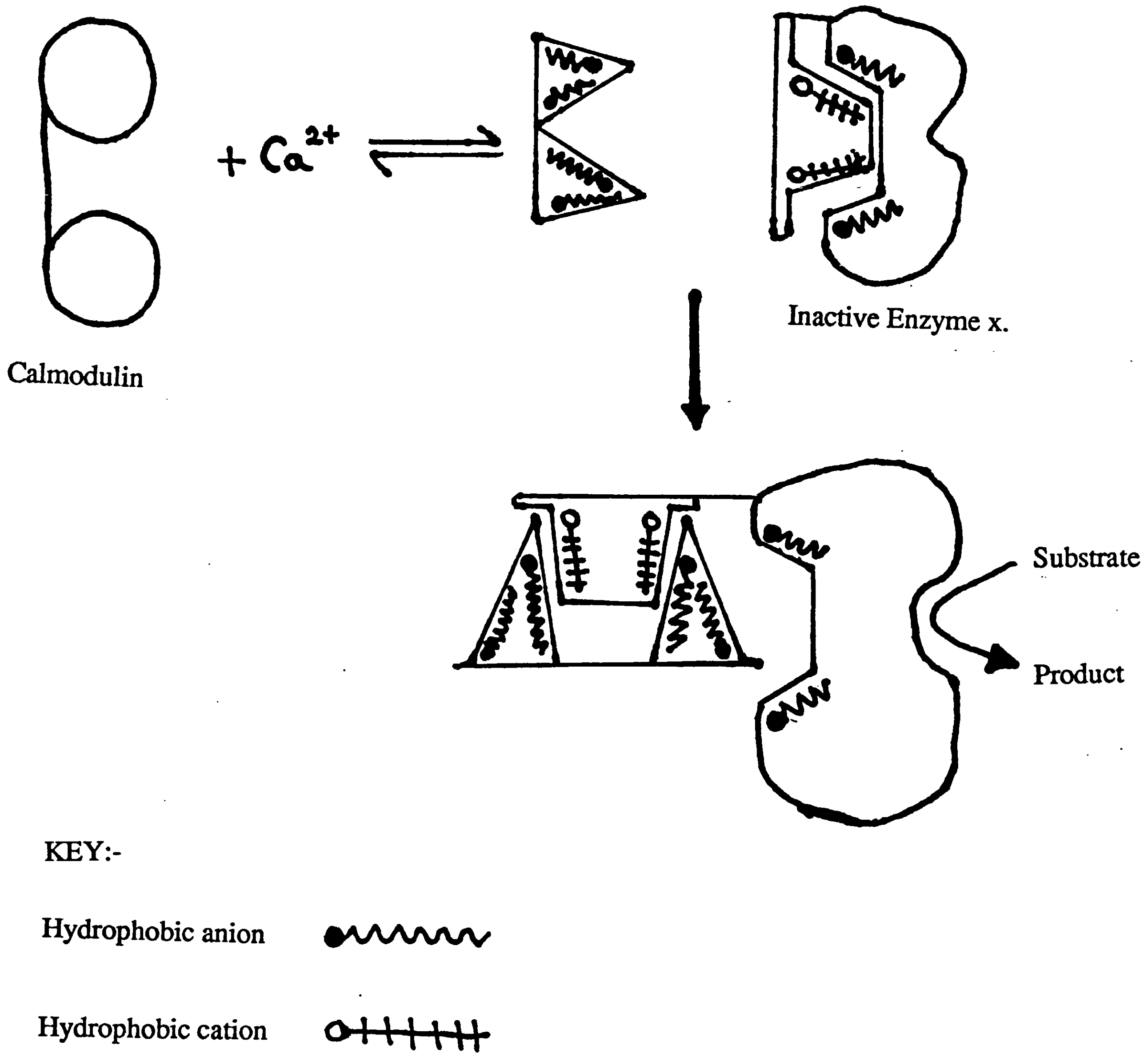


Figure 4. The Flip Flop Model Of Calmodulin Interaction

In this model the binding of calcium to calmodulin results in exposure of hydrophobic domains. These hydrophobic domains can then interact with complementary hydrophobic domains on the target protein so converting it from an inactive form to an active form (Jarret & Madhaven 1991).

proteins. Two notable models are the "Flip Flop Model" and "The Pseudo substrate model" (Jarret & Madhaven 1991). The Flip flop model is shown in Figure 4. The pseudo substrate model is a specialised model to account for the activation of protein kinases. It states that the calmodulin binding site has an amino acid sequence that is similar to the sequence of the site on protein substrates normally phosphorylated by the protein kinase. The calmodulin binding site binds to the active centre of the protein kinase thus blocking catalysis in the absence of calmodulin. Both models describe a dynamic interaction in which calcium binding causes a "flip-flop" between two possible states (Jarret & Madhaven 1991).

Calmodulin is also found as a permanent regulatory sub-unit of its target proteins, for example phosphorylase kinase (Cohen et al. 1978). Here calmodulin forms the delta subunit of the protein. See Figure 5.

There are a large number of inhibitors of calmodulin action. Most are hydrophobic antipsychotics, which include the phenothiazines and their derivatives (Ruben 1983; Jarret & Madhaven 1991). These inhibitors bind calmodulin in the presence of calcium when the hydrophobic residues are exposed on the surface of the calmodulin molecule. The proposed mechanism of interaction is shown in Figure 6. Obviously any calcium chelators will also inhibit the action of calmodulin, e.g. EGTA and EDTA. Likewise any chemicals, e.g. napthalenesulfonamide derivatives such as W-7, that can mimic the hydrophobic calmodulin binding site will be able to bind to the target enzymes thereby preventing calmodulin from interacting and regulating the protein's activity (Hidaka & Tanaka 1982).

1.1.1.1 CALMODULIN BINDING PROTEINS

There are a wide range of proteins that are regulated by calmodulin. These include

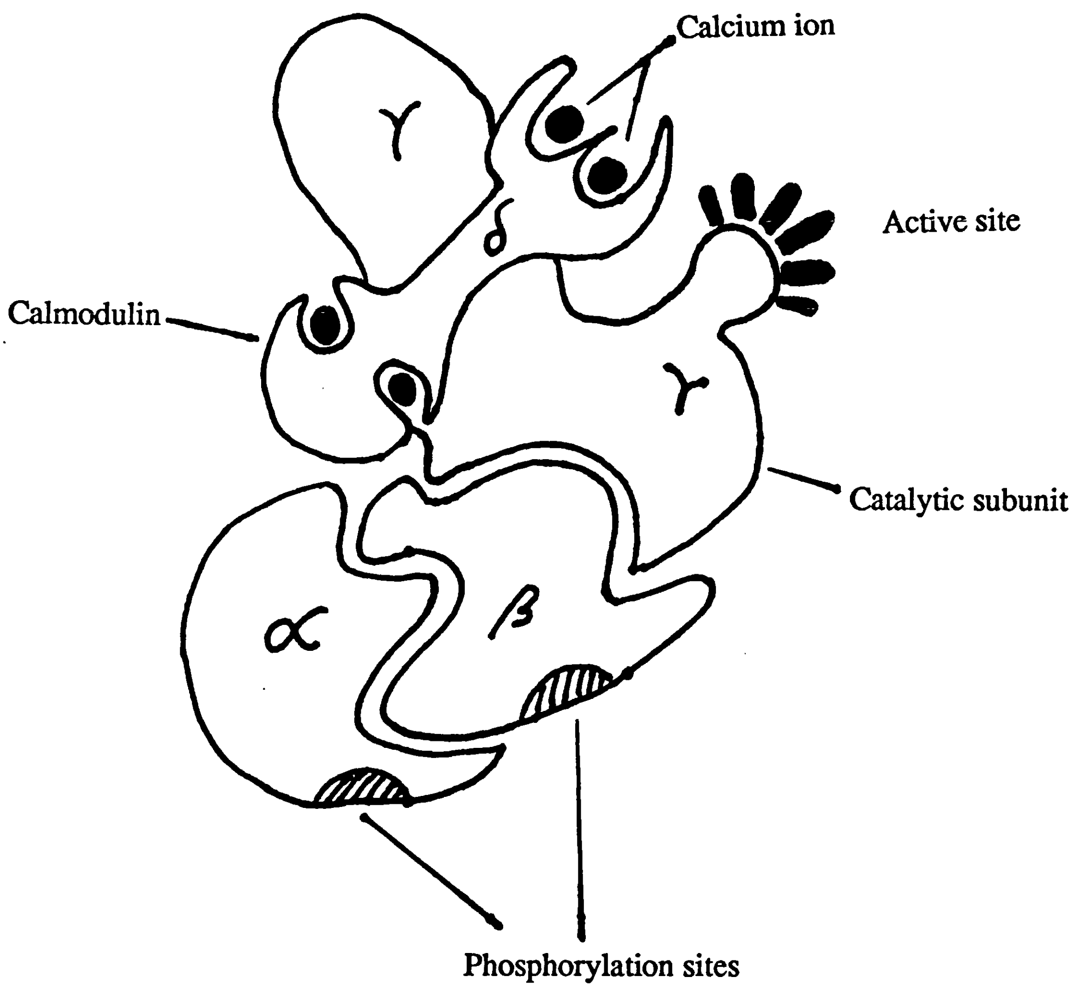


Figure 5. Phosphorylase kinase

Calmodulin forms the delta subunit of phosphorylase kinase, which has a tetrameric structure of $(\alpha\beta\gamma\delta)_4$. Reprinted with permission from "Molecular Biology Of The Cell" 2nd. Edition. Copyright ©1989 B.Alberts et al. Reprinted By Permission Of Garland Publishing Inc.

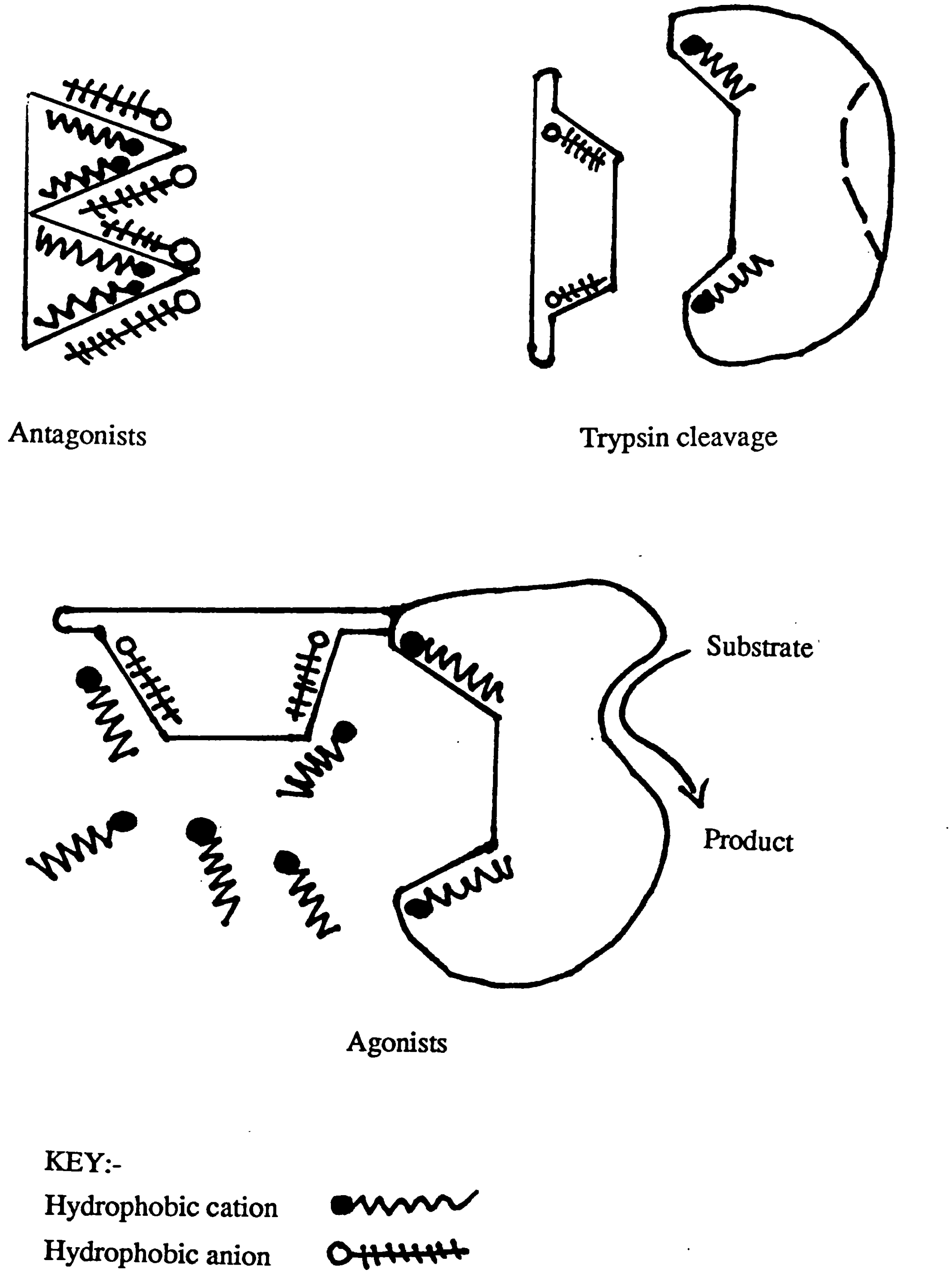


Figure 6. Mechanism Of Inhibition Of Calmodulin

Calmodulin antagonists contain complementary hydrophobic domains that can interact with calmodulin and so prevent interaction with its target domains. Agonists mimic the hydrophobic sites present on calmodulin and interact with the target protein so preventing calmodulin from binding. Finally proteolytic cleavage of some target proteins results in irreversible stimulation (Jarret & Madhaven 1991).

nucleotide phosphatases, protein kinases, phopholipases, phosphodiesterases, methyl transferases, nitric oxide synthase, tryptophan 5' monooxygenase, succinate dehydrogenase, and phosphorylases (Means & Dedman 1980; Ruben 1983; Meijer & Wallace 1985, Paudel & Carson 1990; Jarret & Madhaven 1991; Ikura et al. 1992). These are involved in diverse cellular processes, as is explained below.

1.1.1.1.1 Multifunctional calcium/calmodulin protein kinase

The most ubiquitous enzyme controlled by calmodulin is the multifunctional calcium/calmodulin protein kinase that phosphorylates proteins at serine and threonine residues. It is involved in the regulation of neurotransmitter synthesis and release, carbohydrate metabolism, nuclear envelope breakdown (during mitosis), and neuronal plasticity (MacNicoll, Jefferson & Schulman 1990). It is also the major kinase involved in the co-ordination of cellular responses to hormones and neurotransmitters, which increase the calcium levels by influx via ligand-gated channels and by voltage sensitive channels. Protein kinase is also involved in the regulation of intracellular calcium levels by its interactions with calcium channels, the Ca^{2+}ATPase and inositol 1,4,5-triphosphate and caffeine sensitive stores. It is also able to respond to a narrow working range of calcium concentrations by using calmodulin as the sensor (Homma, Burns & Harris 1990; Schulman 1992).

This protein kinase is unusual as it can autophosphorylate itself at the "autonomy site", also known as the autoinhibitory site as shown in Figure 7 (MacNicoll, Jefferson & Schulman 1990; Schulman 1992). If this site is phosphorylated, then calmodulin remains associated for up to 100 seconds. However when it is not phosphorylated then calmodulin is released within 24 seconds. Thus the rate of dissociation of

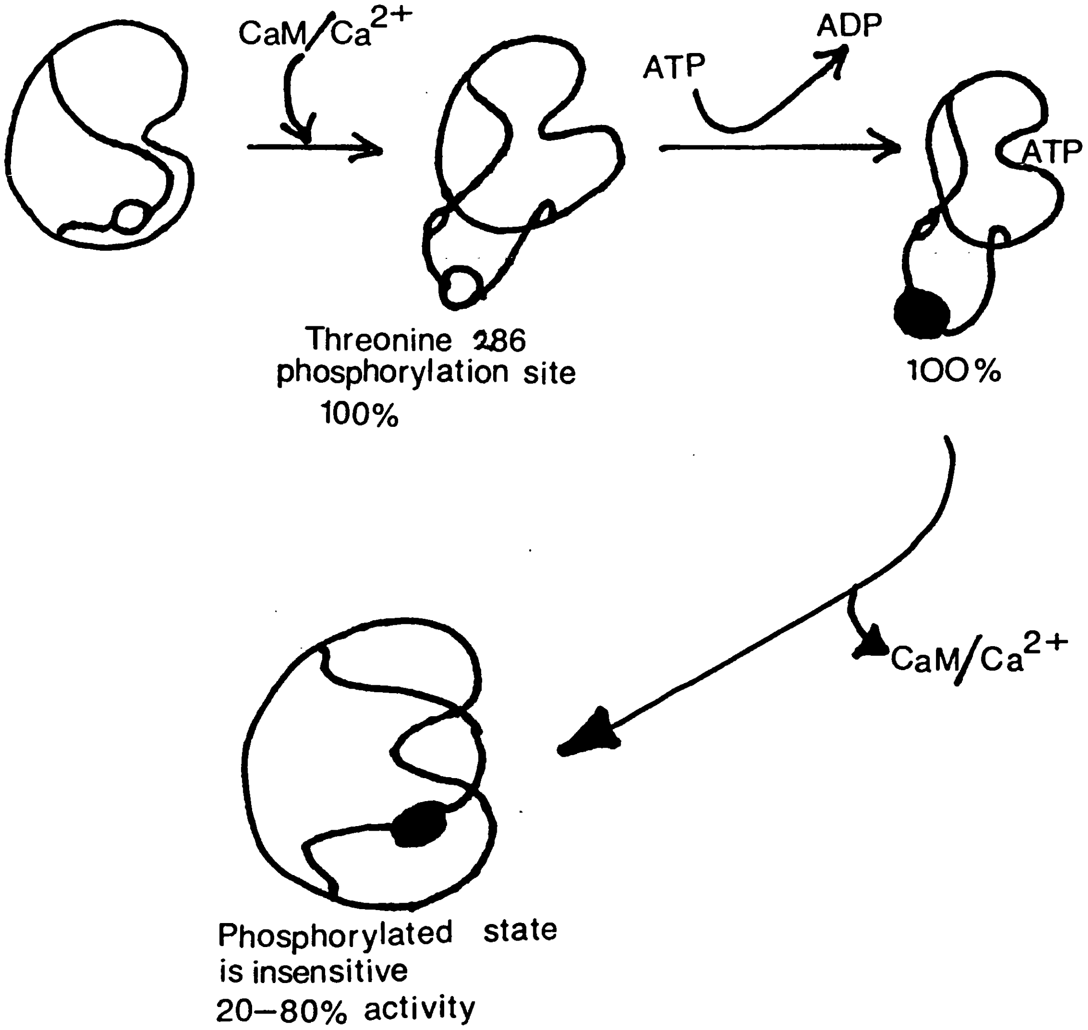


Figure 7. Diagrammatic Representation Of The Phosphorylation Of The Ca^{2+}/Calmodulin Protein Kinase

Binding of calmodulin to protein kinase activates the enzyme, however calmodulin will only remain associated for 24 seconds. If the protein kinase is phosphorylated at threonine 286, then calmodulin is retained for up to 100 seconds. Release of calmodulin from the kinase results in eventual inactivation (Schulman 1992).

calmodulin is phosphorylation dependent. Consequently autophosphorylation is essential for trapping calmodulin. The increased length of time that calmodulin is bound to the enzyme effectively increases the time that the enzyme remains active. Thus allowing the protein kinase a sort of 'memory' that allows it to continue phosphorylating its substrates long after the transient calcium signal that initiated it (Schulman 1992).

The multifunctional protein kinase has acquired many names depending on its tissue source. Kinase from the liver or muscle has been called calmodulin dependent glycogen synthase, whilst that from the brain has been referred to as calmodulin dependent protein kinase I, II and III, synapsin 1-kinase and tubulin kinase. It has a subunit molecular weight of between 37 and 63kD. These can form and enzyme containing between 6-12 subunits with a molecular weight of around 130kD (Manalan & Klee 1984; Nairn, Hemmings & Greengard 1985).

Fodrin (also called calspectin) and spectrin are substrates for the calmodulin regulated protein kinase. These proteins form part of the microfilaments and interact with actin (Manalan & Klee 1984). The calmodulin multifunctional protein kinase has two other substrates that are worthy of note, tyrosine and tryptophan hydroxylase. Tyrosine hydroxylase is activated by cAMP-dependent protein kinase and calcium/calmodulin dependent kinase whereas tryptophan hydroxylase is only stimulated by the calmodulin multifunctional protein kinase. Tyrosine and tryptophan hydroxylase catalyse the first and rate limiting step of the biosynthesis of monoamine neurotransmitters and hormones such as catecholamines (dopamine, norepinephrine and epinephrine) and indoleamines (serotonin and melatonin). Both enzymes require tetrahydrobiopterin as a cofactor (Nagatsu et al. 1985).

2.1.1.1.2 NAD Kinase, Phosphorylase Kinase And Myosin Light Chain Kinase

In plants NAD kinase is completely inactive without calmodulin. The enzyme controls the ratio of NAD/NADP in chloroplasts catalysing the following reaction:

$$NAD + Mg^{2+} ATP \rightarrow \quad NADP^{+} + ADP^{+}$$

(Anderson & Cormier 1978; Cormier, Harman, Putman-Evans 1985). Evidence for the activation of NAD kinase has also been found in sea urchin eggs. During fertilization there is a rise in the concentration of cellular NADP that corresponds to a rise in calcium. Furthermore, calmodulin has been shown to stimulate the sea urchin NAD kinase and inhibitors of calmodulin inhibit the activation of NAD kinase (Epel et al. 1981).

Phosphorylase kinase is unique in having calmodulin as a permanent regulatory subunit. It phosphorylates a single serine residue on glycogen phosphorylase converting it from the inactive *b* form to the active *a* form and thereby controls glycolysis. Phosphorylase kinase has a tetrameric structure, $(\alpha\beta\gamma\delta)_4$ with a total molecular weight of 1,300kD. The subunits have molecular weights of α=145 kD, β= 128 kD, γ=45 kD, δ= 17kD. Its activity is totally dependent on calcium although it can be stimulated by the cAMP dependent protein kinase, which phosphorylates one site on the α subunit and one site on the β-subunit. As well as having calmodulin as a subunit it is also capable of binding one additional molecule of calmodulin, which can activate the enzyme a further 4 fold (Cohen 1980).

Myosin light chain kinase catalyses the transfer of P_i from ATP to the regulatory light chain of myosin, thereby regulating actin-myosin contractions in smooth muscle.

Myosin light chain kinase consists of a single polypeptide, with a molecular weight between 77 and 150kD and its activation is Ca^{2+}/Calmodulin dependent (Klee & Vanaman 1982; Manalan & Klee 1984).

1.1.1.1.3 Calmodulin Dependent Protein Phosphatases

There is one major calcium/calmodulin dependent phosphatase, calcineurin. It has also been referred to as phosphoprotein phosphatase, as inhibitor protein, as modulator binding protein 1, as Type 2b phosphatase and CaM-80. This phosphatase is predominant in neural tissue and is the major calmodulin binding protein in bovine brain (Klee, Crouch & Krinks 1979; Kincaid & Vaughnan 1986; Tallant & Cheung 1986; Klee et al. 1992).

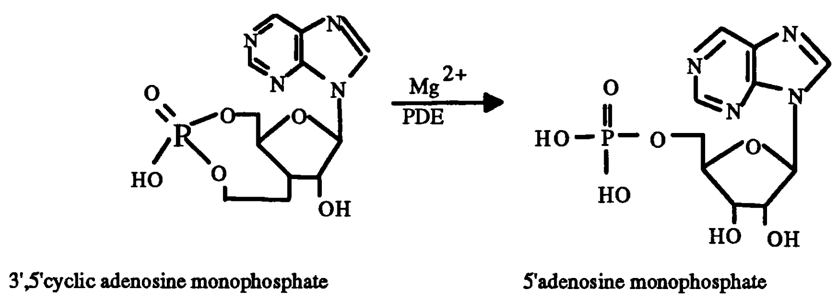
Calcineurin is a serine/threonine phosphatase with a narrow substrate specificity. Activation of calcineurin by calcium/calmodulin is in the range of 0.1-1.25μM calcium (Klee et al. 1992). One of its substrates is the alpha subunit of phosphorylase kinase. In both liver and skeletal muscle, calcineurin accounts for 60% of the phosphatase activity toward inhibitor-1 and phosphorylase kinase. Interestingly it was first isolated as an inhibitor of calmodulin-dependent phosphodiesterase (Tallant & Cheung 1986).

Calcineurin is a globular protein with a molecular weight of 80kD, by gel filtration. It is a heterodimer with an α-subunit of $\approx$60kD an β–subunit of $\approx$19kD. The α-subunit interacts with the calcium/calmodulin complex and contains the catalytic site. However, the beta subunit is also a calcium binding protein, which will bind four calcium ions and is composed of 168 amino acids. Like calmodulin it possesses the characteristic EF-hand structure seen in the calmodulin family of calcium binding proteins. It also has a high number of acidic residues, no tryptophan or cysteine residues and its amino terminus is blocked by acylation with myristic acid (Tallant & Cheung 1986).

1.1.1.1.4 Calmodulin Dependent Phosphodiesterase

The calcium dependent phosphodiesterase is one of many multiple forms of cyclic nucleotide phosphodiesterases. It is of key importance to the calmodulin story, because it was while studies were carried out on this enzyme that calmodulin was first discovered in the late 1960's by W.Y.Cheung (1980).

Phosphodiesterase (PDE) is found in both the cytosol and membrane fractions of cells, and its distribution varies according to tissue and species. It is a homodimer, with a subunit molecular weight of 57-60kD (Manalan & Klee 1984). PDE represents about 1-3% of the calmodulin binding proteins present in bovine brain, and catalyzes the hydrolysis of the ribose 3'5'-phosphodiester bonds as shown below.



It is specific for cAMP and cGMP however it will hydrolyze cUMP, with stimulation by calcium/calmodulin increasing its activity by up to 50% (Lin & Cheung 1980). The apparent affinity of PDE for calmodulin in the presence of 300mM calcium is 1nM (Kincaid & Vaughnan 1986).

1.1.1.1.5 Calmodulin Dependent Adenylate Cyclase

There are eight forms of adenylate cyclase present in mammalian systems, two of which are calmodulin sensitive, type I and type III. Type I calmodulin sensitive adenylate cyclase is thought to be important for learning and memory. A Drosophila learning mutant, rutabaga is deficient in type I adenylate cyclase. Type I adenylate cyclase is found only in the brain and in the retina of rats; here it is located within specific regions that are implicated in memory including the neocortex, hippocampus and the olfactory system. Type III adenylate cyclase is not as sensitive to calmodulin as type I. Both forms are inhibited by other calmodulin target proteins such as calcineurin (Storm, Xia & Choi 1992).

Adenylate cyclases are transmembrane glycoproteins and have a molecular weight of 135kD. Calmodulin interacts with adenylate cyclases in a 1:1 ratio (Storm, Xia & Choi 1992).

Bordetella pertussia produces an extracellular adenylate cyclase that is stimulated by calmodulin in the presence or absence of calcium. This is particularly noteworthy as Bordetella pertussia does not contain calmodulin. Likewise anthrax toxin also contains a calmodulin sensitive adenylate cyclase. Therefore it has been suggested that these extracellular cyclases may be a "bacterial toxin" that is activated, on entry into host cells, by calmodulin and disrupts normal cellular processing by increasing the cellular levels of cAMP (Manalan & Klee 1984).

1.1.1.1.6 Phospholipase A2 and C

In the venom of Naja naja there is a phospholipase A2 that is stimulated by calmodulin. However, phospholipase A2 from other sources such as pancreas is calcium/calmodulin independent (Craven & DeRubertis 1985).

There is evidence for a calcium/calmodulin sensitive phospholipase in both the kidney and lung. In the inner medulla of the kidney it is believed that calmodulin regulates the release of arachidonate from tissue lipid stores, for prostaglandin synthesis. It is known that the action of vasopressin, which increases the rate of prostaglandin synthesis, is a calcium/calmodulin dependent process (Craven & DeRubertis 1985). There is also evidence in lung that different modes of transmembrane calcium shifts, trigger the onset of the arachidonic acid cascade using a calcium/calmodulin link, which is thought to be phospholipase (Seger & Suttorp 1985).

1.1.1.1.7 Calcium/ Calmodulin ATPases And Axonemal ATPases

Calmodulin is involved in the regulation of intracellular levels of calcium, by its interaction with calcium channels; Ca^{2+}ATPase and inositol 1,4,5 triphosphate sensitive calcium release from intracellular stores (Homma, Burns & Harris 1990). The extracellular fluid of most organisms has a calcium concentration of 1mM, whilst the intracellular calcium is 10^{-7}M. This represents a 10,000 fold concentration gradient that is maintained by the enzymatic hydrolysis of ATP.

The regulation of the Ca^{2+}/Mg^{2+}ATPase by calmodulin is to terminate calcium messages and to return the intracellular calcium concentration to resting levels. Addition of purified calmodulin, *in vitro,* results in a 3-4 fold increase in ATPase activity. For example, in red blood cells the calcium ATPase has a low rate of activity, which increases by four-fold when activated by calmodulin (Vincenzi & Hinds 1980).

Eukaryotes have two distinct filament-based systems for ATPase driven processes.

The first is the actomyosin system where calmodulin mediates its effects through myosin light chain kinase, and the second system is the dynein-microtubule system responsible for ciliary and flagellary movement. Calcium and calmodulin have been determined to regulate the rate of activity of the dynein ATPase in Tetrahymenia pyriformis, Chlamydomonas rheinhardthii, sea urchin eggs and Paramecium aurelia (Klee & Vanaman 1982; Manalan & Klee 1984).

1.1.1.1.8 Neurogranin And Neuromodulin

Neuromodulin and neurogranin are two calmodulin binding phosphoproteins that are substrates for protein kinase C. Neuromodulin is found in the presynaptic membrane and has also been called GAP43, B50, F1, p57 and pp46. Its synthesis is strongly correlated with axon outgrowth suggesting that it is involved in the general mechanisms that control nerve growth during development and regeneration. The correlation between neuromodulin phosphorylation and the persistence of long term potentiation also indicates that neuromodulin may play a role in both normal neurite growth, in developing brain, and neural plasticity at adult synapses. Several other functions have been proposed for neuromodulin: sequestration of calmodulin along the cytoplasmic surface of membranes, the modulation of neurotransmitter release and the regulation of GTP binding to the G_o protein.

Neurogranin (p17) has a molecular weight of between 15 and 19kD on SDS-PAGE depending on the percentage gel used. The phosphorylation of neurogranin by protein kinase C is inhibited by the interaction of calmodulin with neurogranin, as the phosphorylation site falls within the calmodulin binding domain. Neurogranin is essentially located post synaptically whereas neuromodulin is exclusively presynaptically and confined to axons. It has therefore been proposed that neuromodulin serves to localize calmodulin at the neuronal cell membrane and releases the calmodulin in response to increase in intracellular calcium concentration and/or to phosphorylation by protein kinase C (Baudier et al. 1991).

1.1.1.2 Cellular Processes Involving Calmodulin

There is a large body of evidence to support the involvement of calmodulin in nuclear processing, e.g. DNA repair, expression of genes, phosphorylation and dephosphorylation of nuclear proteins, the activity of an actin-linked contractile system, as a trigger for DNA replication, possibly the condensation/relaxation of chromatin and the fragmentation of DNA during apopstasis (Bachs et al. 1990; Bachs 1992). This is supported by the growing number of calmodulin binding proteins that are being isolated from the nucleus.

Calmodulin also regulates the assembly/dissassembly of the cytoskeleton including actin containing microfilaments and microtubules (Hidaka & Sasaka 1985; Klee & Vanaman 1982). Calmodulin may regulate microtubule assembly-dissassembly by two mechanisms. Firstly it may stimulate the phosphorylation of stable tubule only polypeptides (STOPS) which may promote the depolymerization of microtubules. Secondly calmodulin may interact directly with microtubule associated proteins (MAPS) and prevent the MAPS stimulation of microtubule nucleation. Also MAP-2 and τau factor have been shown to contain multiple phosphorylation sites for a calmodulin dependent protein kinase. Once phosphorylated the MAPS no longer stimulate tubulin polymerization (Klee & Manalan 1984). Overall though calmodulin serves to inhibit microtubule polymerization.

Another protein that interacts with calmodulin is caldesmon. In the absence of calcium, caldesmon binds to actin and inhibits the actin-myosin interaction. In the presence of calcium and calmodulin, caldesmon preferentially binds to calmodulin and so the actin-myosin complex can form, resulting in activation of the actomyosin ATPase (Manalan & Klee 1984).

In Echinoderm sperm there is a reaction termed the 'acrosome reaction'. It occurs when a sperm contacts the echinoderm ovum. Here, the elongation of an acrosomal process from the apical part of the sperm head occurs, caused by the polymerization of actin. This reaction is calcium dependent and the calmodulin is localized near the acrosome, but is released from the sperm once the acrosome reaction is complete. As calmodulin serves to cause depolymerization of microtubules it seems unlikely that calmodulin is responsible for triggering the polymerisation process. This leaves two possible roles for calmodulin; either it is required for the fusion process, or it is needed for the activation of the ovum which occurs when calcium and calmodulin act as a 'calcium bomb' (Sano 1985). It does however seem possible that calmodulin acts to maintain the actin in a depolymerized state until the sperm makes contact with an ovum.

1.1.1.3 Calmodulin Related Defects

Due to its wide involvement in cellular processing it is hardly surprising that decreased levels of calmodulin have been found to be associated with mineral corticoid induced hypertension, degenerative disease of the retina and experimental diabetes. There is a 2-3 fold increase in calmodulin levels in viral or chemically induced cellular transformation (Tshudi et al. 1985). This supports claims that calmodulin has a role in triggering DNA replication.

In Aspergillus nidulans, a filamentous fungus, there is one copy of the calmodulin gene. If this is mutated or deleted, it is lethal and the fungus cannot survive.

Moreover, overexpression of this gene not only increases the cell growth rate but also its requirement for calcium (Means 1992).

In cystic fibrosis patients it has been found that the activity of the Ca^{2+}/Mg^{2+} ATPase is decreased. The decrease in activity has been correlated with the severity of the disease. The implication here is that there is some general defect in calcium transport and/or signals in these patients red blood cells and the secretory cells of the lung (Vincenzi & Hinds 1980).

In apples a physiological disorder called Bitter Pit appears to be due to a failure of calmodulin activation. The target site appears to be the fruit membranes and there is an accompanying deficiency of calcium (Fukumoto 1985).

1.1.2 CALMODULIN GENETICS

Currently over 25 amino acid sequences are known for calmodulin and all show between 80-100% sequence homology. Furthermore, the nucleotide sequence for calmodulin has been shown to be equally well conserved, with between 75-95% sequence homology for the calcium binding sites and an overall homology of 70-80% (Zimmer et al. 1988; Putkey et al. 1983). It is therefore, a highly conserved protein, which supports its central role in cellular processes.

As calmodulin is highly conserved it may be important for higher organisms to contain multiple (active) genes to be protected against deleterious mutations that are lethal. Multigene families exist for other proteins and encode multiple isoforms that have subtle differences in the primary structure. In some species there are also pseudogenes that are expressed to mRNA level, but it is not known if these are expressed as proteins. Multigene families for calmodulin have been found to exist in rats, frogs, trypanosomes, humans and possibly chicken. See Table 3.

ORGANISM	N°. GENES	FEATURES	WORKERS
Plasmodium falciparum	1	Present on chromosome 14	Robson & Jennings 1991
Trypanosomes	3	Polygene transcripts are tandemly arrayed and all actively transcribed.	Tschudie et al. 1985
Dictyostelium discoidium	1		Goldhagen & Clarke 1986
Chlamydomonas	1		Zimmer 1988
Drosophila melangaster	1	In region 49A of chromosome 2R.	Smith et al. 1987
Yeast	1	Locus resides on chromosome II	Davies et al. 1986, Takeda & Yamamoto 1986
Ceanorhabdatis elegans	1		Salvato et al. 1986
Sea urchin, Arabicia punctulata	2	Duplication of the entire genome	Hardy et al. 1988
Xenopus laevis	2	Duplication of gene at two loci	Chein & Dawid 1984
Chicken	1 (2?)	Multiple species of RNA derived during transcriptoion from extra 3'non-coding regions. Also has one intronless gene.	Leglace 1983
Mouse	2		Bender et al. 1988
Rat	3	Plus one pseudogene	Nojima, Kishi & Sokabe 1987
Homo sapiens	3	Plus retropseudogenes.	Wawrzynczac & Perham 1988, SenGupta et al. 1987, Fischer et al. 1988.

Table 3 Gene Distribution For Calmodulin

This shows the number of genes that have been determined for the various organisms shown together with any special features.

The intron/exon structure of some of the calmodulin genes is shown in Figure 8. On the basis of the standard exon shuffling model, which states that exons correspond to functional domains of proteins that can be reshuffled by recombination to create novel proteins, and intramolecular homology the calmodulin gene would be expected to possess four exons in the coding region with each exon encoding one calcium binding domain. However there is a wide range of coding exons varying from one to six (Nojima & Sokabe 1987). However, the actual location of the introns has been highly conserved throughout the evolution of the calmodulin gene. The first noncoding exon containing the ATG translation codon is separated from the other coding exons by the first intron. This is also the only intron found in the calmodulin gene from Schizosaccharomyces pombe. Other calcium binding proteins with four calcium-binding sites, e.g. troponin C, also possess this intron and in the same position. This indicates a common ancestral gene (Koller, Schnyder & Strehler 1990). However, the Plasmodium falciparium gene does not conform with other calmodulin genes because it only contains one intron that does not separate the initial ATG from the remaining coding sequence (Robson & Jennings 1991).

The intron between exons 3 and 4 is also present in the genes of other calcium binding proteins, but has not been conserved throughout evolution because in some cases it has been lost, e.g. the rat calmodulin II gene. Here, there are also differences in the length of the introns as can be seen between exons 5 and 6 in Figure 8 (Koller, Schnyder & Strehler 1990).

In evolutionary terms it would appear that the separation of the calmodulin genes took place before the branching of the avian/mammalian lineage's, assuming that the degree of sequence identity in noncoding regions correlates with a timescale (Koller, Schnyder & Strehler 1990).

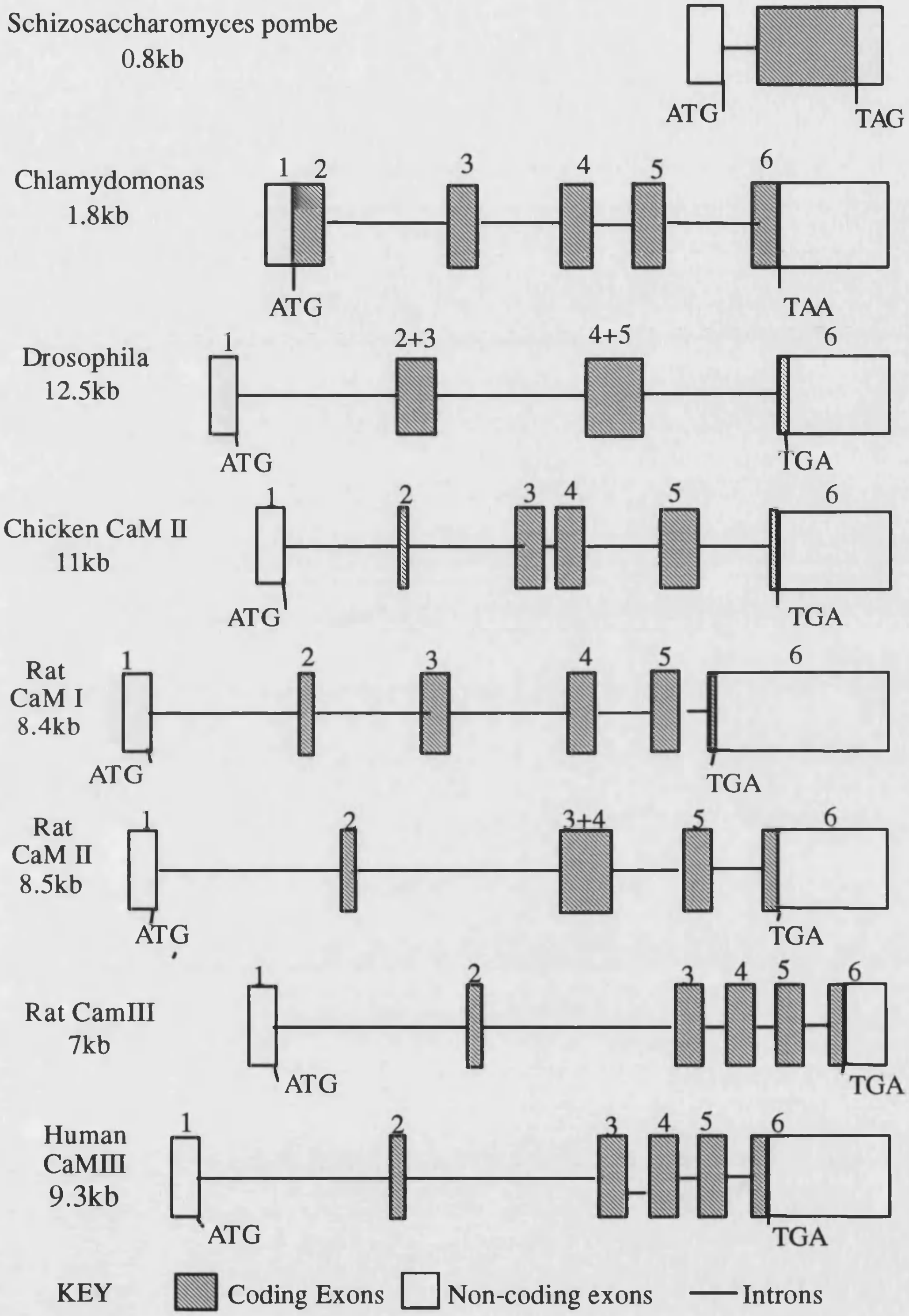


Figure 8 Intron/Exon Structure Of Calmodulin Genes

This shows the intron/exon strucure of calmodulin genes of different species (Koller, Schnyder & Strehler 1990). N.B. This is not drawn to scale.

1.2 CESTODES

Cestodes or tapeworms belong to the phylum Platyhelminthes, class Cestoidea. There are many orders within this group, but the one in which the most important parasitic tapeworms of man and domestic animals occur is the order Cyclophyllidea (Noble & Noble 1982). Within this order there are two major families, the Hymenolepididea and the Taeniidae. Members of the family Hymenolepididea infect mostly birds and some mammals. The Taeniidae contain the most medically important species, including Echinococcus granulosus, Taenia saginata and T.solium, the beef and pork tapeworm respectively.

The adult worm of the Cyclophyllidea is composed of a 'head' bearing an array of hooks and suckers (referred to as the scolex), a neck region and then a body composed of segments called proglottides. See figure 9. They have no alimentary canal or circulatory system so that all exchange of metabolites is across the unique tegument of the cestode. In fact they are armed with a host of uptake mechanisms, even to the extent of adsorbing host enzymes onto their surface and using them to break "food" down into its components, which the worm can then absorb (Smyth 1985).

Tapeworms (Cyclophyllidea) are a particular problem in developing countries where transmission is aided by poorer standards of sanitation. However in the Western world, there is an increase in the levels of infection in domestic animals. One example of a particularly problematic tapeworm is Echinococcus granulosus which is the causative agent of hydatid disease or hydatidosis. The adult form of this worm resides in the intestines of its definitive host, which is usually a member of the dog family and it is the intermediary stage of this worm that causes the disease. The

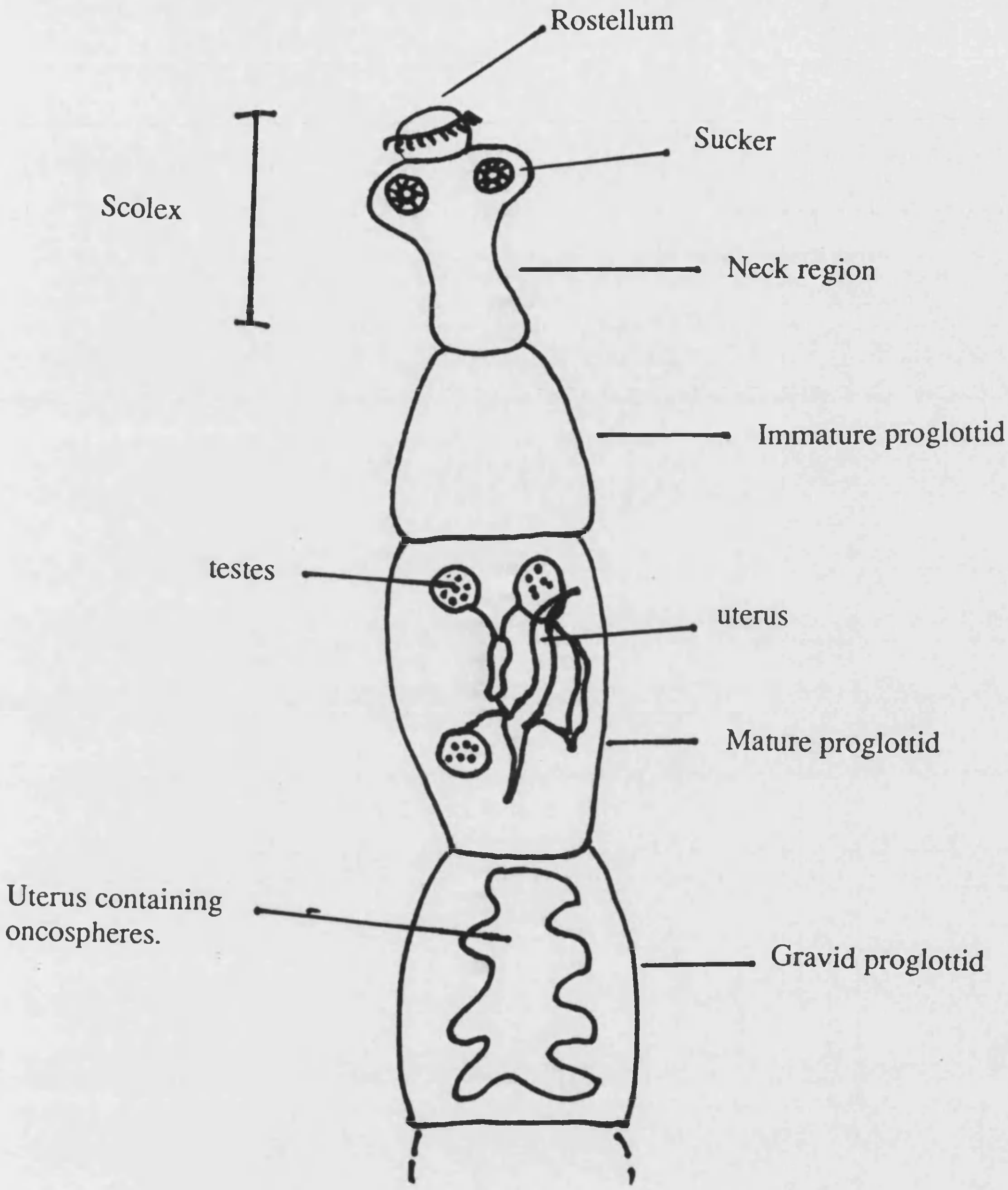


Figure 9. Generalized Scheme Of A Tapeworm

The scolex forms the 'head' of the tapeworm and bears an array of hooks (rostellum) and suckers. This is connected to the neck region where the tissue is rapidly dividing and differentiating to form proglottides. Further along the tapeworm the proglottides differentiate to contain the reproductive organs. In the terminal proglottides they are packed with oncospheres, and the organ structure is degenerating. These mature proglottides are referred to as gravid.

intermediate host is frequently a human but it can also be herbivores such as cattle or sheep. The eggs or oncospheres, from the adult are released in the faeces, and are then ingested by the intermediary host. The egg develops in the intestinal lumen into a hydatid cyst that is full of protoscoleces. These are capable of either forming another cyst or if eaten by the correct definitive host will mature into the adult worm. As the cyst matures so it gains in size and weight causing immense pressure on the other organs in the abdomen, which causes death. If the definitive host then eats these dead remains it becomes infected, and the cycle is started anew. Surgical removal of the cysts is difficult as its rupture is highly probable resulting in the patient having several new cysts (Smyth 1976). See Figure 10.

Drugs are used to eliminate many species of parasitic tapeworm. These drugs include the benzanilides, e.g. niclosamide; the benzimidazoles, e.g. mebendazole and thiabendazole; the acridine dyes, e.g. mepacrine and the quinolines, e.g. praziquantel. See Figure 11.

The benzanilides are broad spectrum antihelminthics that are used to treat cestode, trematode and nematode infections. Niclosamide interferes with the energy metabolism of helminths probably by inhibiting ATP production as it is a potent uncoupler of oxidative phosphorylation. It also inhibits the tegumental glucose transporter of tapeworms. Ultimately niclosamide causes detachment of the scolex and disintegration of the worm. This drug is also generally used to treat Taenia and Hymenolepis infections where it is approximately 95% effective (Coles 1983; James & Gilles 1985; Hipkiss 1986).

The benzimidazoles exert their effects on tubulin, microtubules, glucose uptake, glycogen depletion and by inhibiting the release of secretory granules which result in cellular degeneration. Benzimidazoles are used for the treatment of hydatidosis

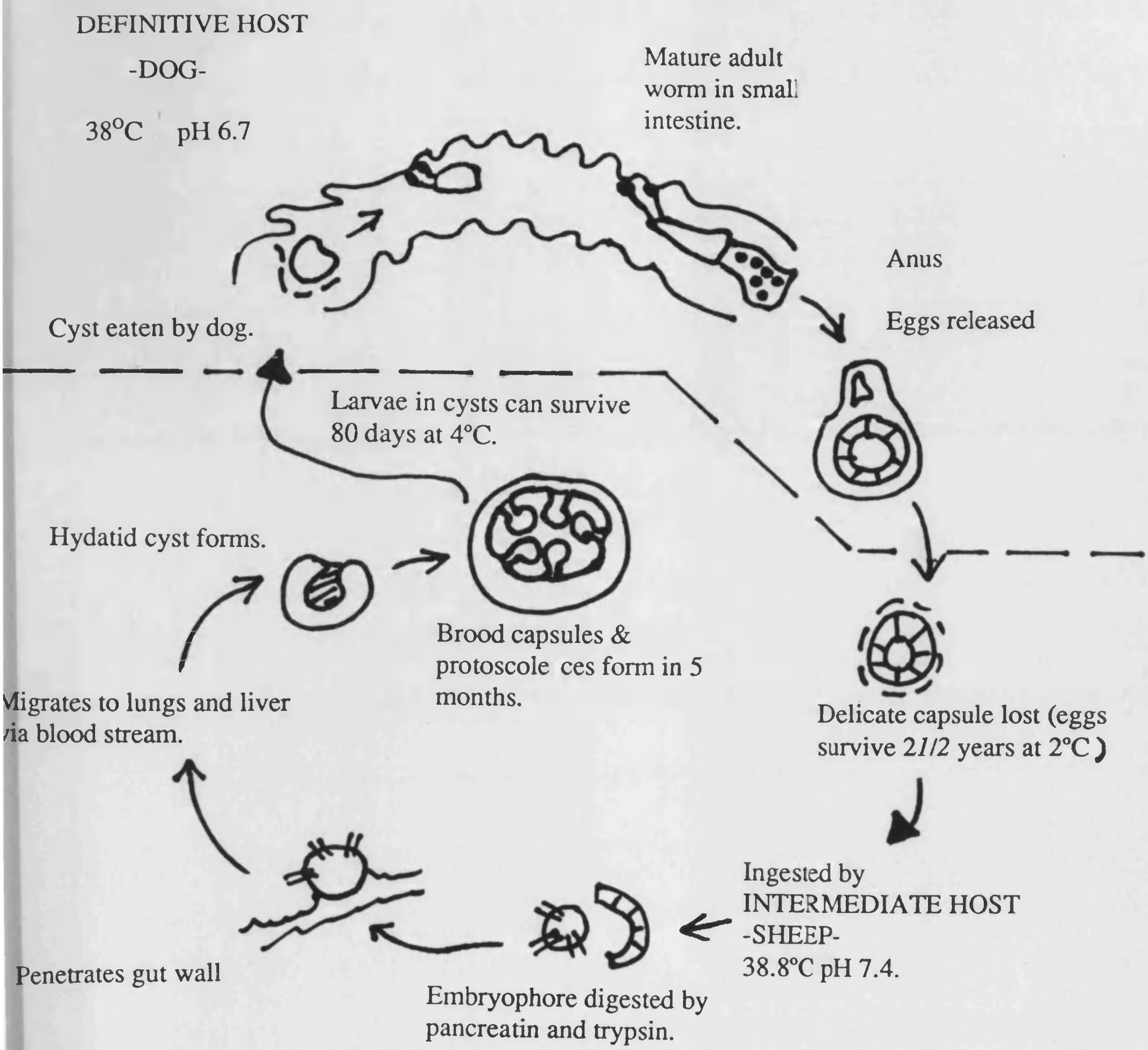
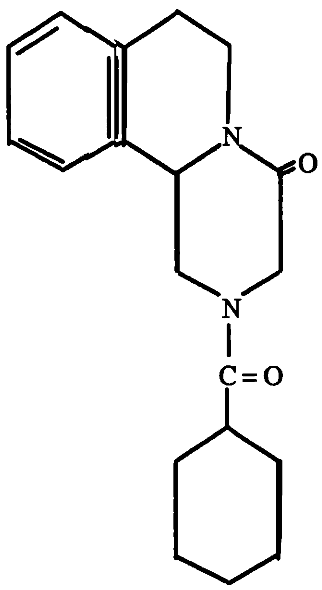


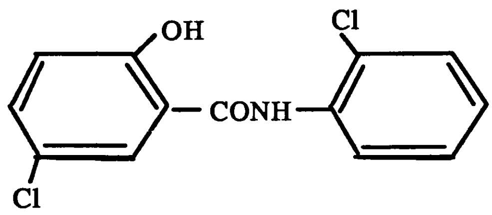
Figure 10. Life Cycle of Echinococcus granulosus.

Eggs are released in the faeces of the definitive host, which are then ingested by the intermediate host, which is generally a herbivore. The egg then develops in the lungs and/or liver to form a hydatid cyst, which contains thousands of protoscoleces each capable of either forming another cyst or of developing into an adult worm. The definitive host, usually a dog eats the cysts containing the protoscoleces, which attach to the gut wall and develop into mature worms.



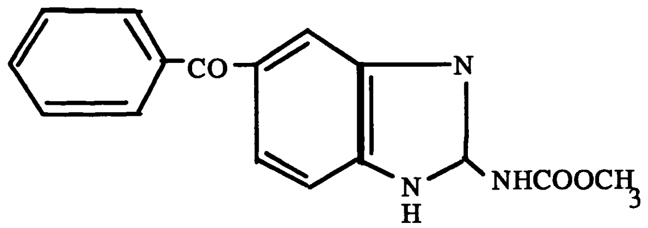
Praziquantel

(A quinoline)



Niclosamide

(A benzanilide)



Mebendazole

(A benzamidazole)

Figure 11. Structures Of The Major Antihelminthics.

This shows the chemical structures of the major drugs used to treat cestodes (Cole 1983).

although they are active against cestodes and nematodes. Treatment is continued over several months and a cure is not guaranteed. This is an added problem in countries such as Africa where people tend to be nomadic. Consequently it is very difficult to give repetitive doses, which makes this an unfavourable drug (Coles 1983; James & Gilles 1985; Hipkiss 1986).

Mepacrine, an acridine dye, inhibits incorporation of glucose in H.diminuta.

Praziquantel, a quinoline, is frequently used for the elimination of schistosomes and for the eradication of tapeworms in both humans and household pets. It causes vacuolisation and disruption of the tegument in most cestodes and schistosomes. In a variety of cestodes, including Hymenolepis diminuta, praziquantel induces a massive contraction of the musculature followed by a large influx of calcium into the worm. This large influx in turn may block the release of inhibitory neurotransmitters such as acetylcholine and possibly stimulate the release of 5'hydroxytryptophan resulting in paralysis of the worm. Praziquantel also inhibits the membrane associated Ca^{2+}ATPase in schistosomes and the NADH-oxidizing enzymes in Ascaris suum. In H.diminuta, praziquantel not only causes a reduction in glucose uptake but also allows an efflux of glucose from the worm, other effects are on increased lactate secretion, and an increased secretion of acidic metabolites. Thus the overall effects of praziquantel are thought to be due to its interference with calcium ion movement (Coles 1983; James & Gilles 1985; Hipkiss 1986).

Praziquantel is also used to treat E.granulosus adult worm infections because it has very few side-effects unlike some of the other drugs. Another advantage is that it can be delivered orally or intramuscularly and only one dose of 5mg/kg of host is needed, which should give a 100% eradication. Mebendazole is only 100% effective against the immature adult worm and has to be given twice in doses of 20mg/kg host. Apart from praziquantel the only other drug that is 100% effective on adult worms is bunamidine given at a dose of 25mg/kg host (Coles 1983).

1.2.1 Hymenolepis diminuta

Family Hymenolepididea, Sub-Family Hymenolepidnae, Genus Hymenolepis, Species Hymenolepis diminuta (Burt 1980).

Hymenolepis diminuta is a common species of tapeworm found in rats, but it will occasionally infect humans. In India approximately 6% of the population are infected with H.diminuta. However its incidence in man is usually less than 1% so it is considered a safe model with which to work.

The life cycle of H.diminuta is shown in Figure 12. The adult resides in the small intestine of the definitive host, which is usually a rodent and produces oncospheres that are shed and released in the faeces. These are then ingested by an intermediary host, which is usually an arthropod, typically Tenebrio molitor or Tribolium confusum. The change in host temperature, coupled with the action of the beetles' mandibles, activates the oncosphere. Once ingested it uses its hooks and releases' substances from the penetration gland to faciliate penetration through the gut wall. It then migrates to the heamocoel where it develops into a cysticercoid, which may take anything from 5 days at 37°C to 65 days at 15°C. Eventually the beetle will be ingested together with its mature cysticercoids by another rodent. During the passage through the stomach the cysticercoids are protected by their outer layers, removal of the cyst wall in the stomach would prove lethal. In the rodent there are several stimuli that trigger the cysticercoid to evaginate and attach to the gut wall. These include the action of chewing, the change in body temperature and the action of the

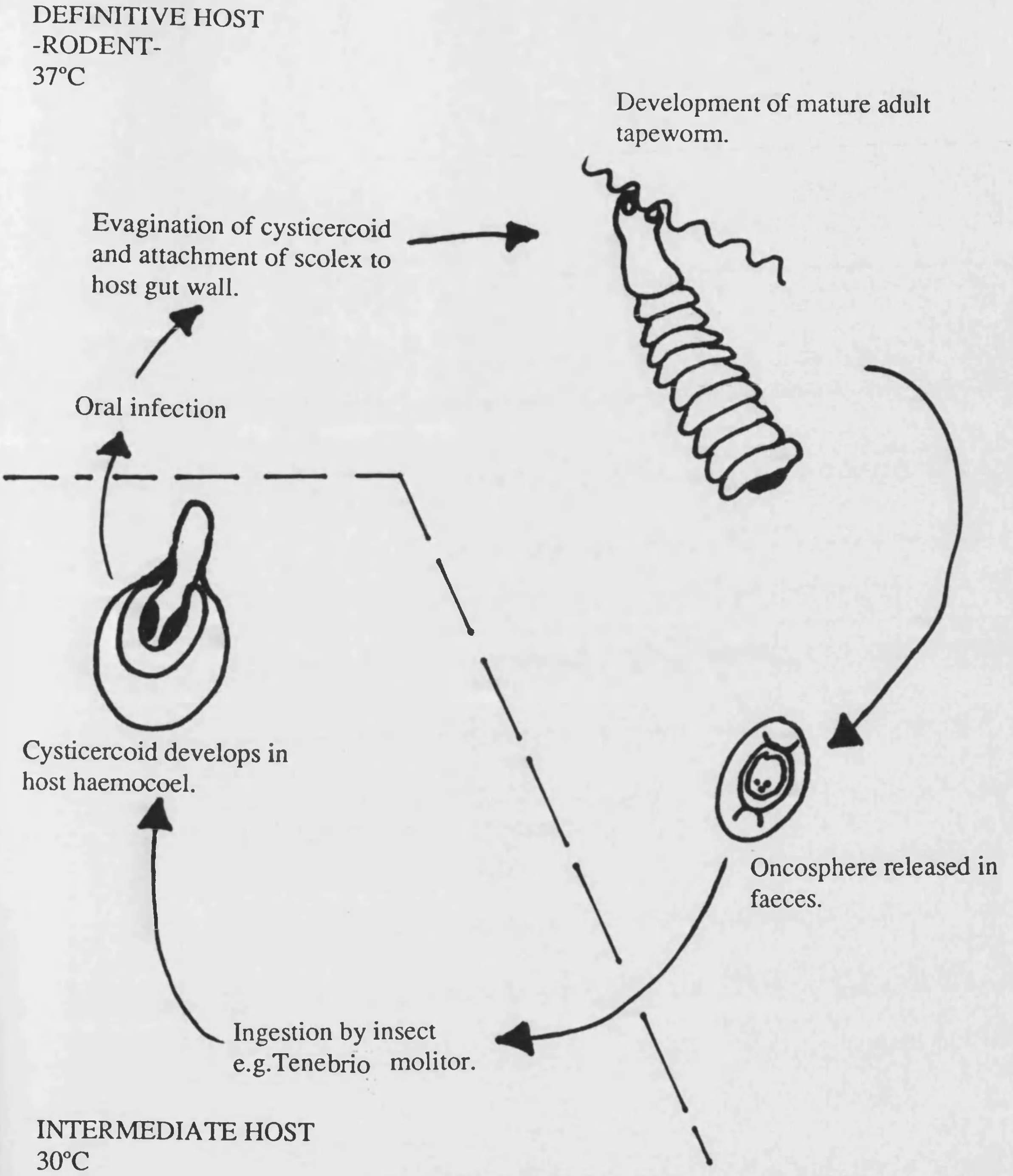


Figure 12. The lifecycle of Hymenolepis diminuta.

Oncospheres are released in the faeces and later ingested by an intermediate host, typically an insect. Within the insect it develops into a cysticercoid in the haemocoel. Eventually the insect will be consumed by a rodent, which is the definitive host. Here the cysticercoid is stimulated to evaginate and attach to the gut wall where it develops into an adult worm.

various bile salts. Once the scolex is attached to gut wall it commences strobilization. Proglottides form from the neck, which is the germinative region, and as they become distanced from the neck they mature. Once the worm has reached its maximum length, new proglottides are only produced in sufficient numbers to replace those lost by apolysis. A mature proglottid is one that is gravid, i.e. full of oncospheres with degenerating reproductive organs. The pre-patent period of the adult worm is between 10 and 24 days with 16-17 days being the average (Roberts 1961; Smyth 1976; Burt 1980). The life span of H.diminuta is as long as its host, which in the case of rats is around 3 years. By artificial passage, worms have been maintained for 14 years (Roberts, Bueding & Orrell 1972).

H.diminuta has a very high egg production producing 8.3 million eggs per year, which is equivalent to 23,000 eggs/day and represents 75μg egg/mg body weight. The oncospheres are viable for up to 6 months providing that the outer capsule has not been damaged. Egg production is affected by the density of the worm infection and is part of the so called 'Crowding effect' (Boddington & Mettrick 1981). This is due to several factors, which include local immunity within the host's intestine, insufficient nutrients, inhibition of worm growth by substances excreted by other worms, and actual physical crowding of space. These factors have a number of effects on an infection of H.diminuta. However, it affects the fecundity of the worm and therefore egg production, and the size of the worms. It has been shown that the size of H.diminuta is inversely proportional to the number of worms in an infection. One of the reasons for these effects is that crowding results in inhibition of DNA, RNA and protein synthesis within the worm. RNA synthesis is inhibited by 60%, and that of protein synthesis by 90% (Roberts 1961; Bolla, Roberts 1971; Quinnel 1988).

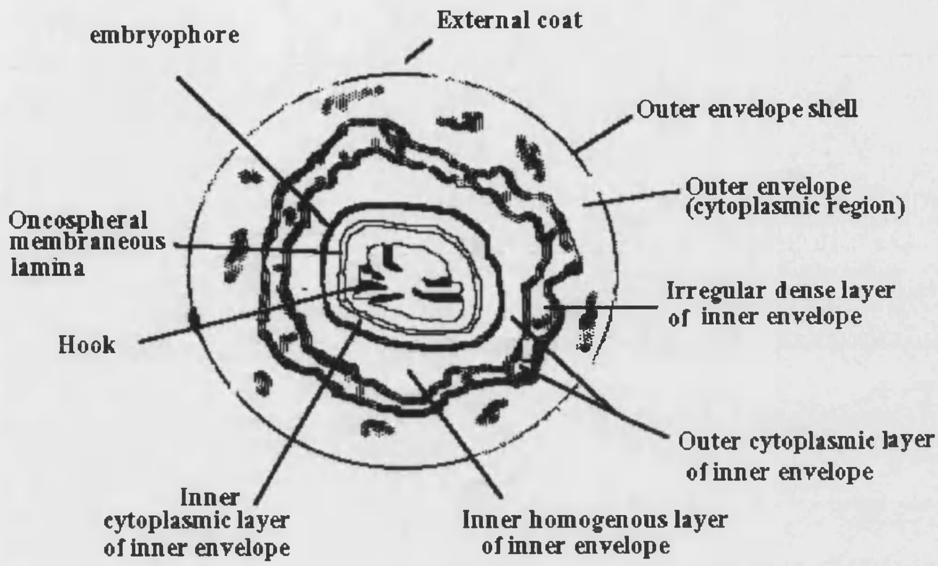
The temperature of the host also has a significant effect on worm size. Hosts maintained in a cold environment suffer from immunosuppression, which results in larger worms (Huebert, Evans & Hardy 1990). In extreme conditions' tapeworms will destrobilate retaining only a few proglottids next to the scolex, thus ensuring their survival. Naturally this reduces the competition between fellow worms for nutrients (Roberts 1961; Bolla & Roberts 1971; Quinnel 1988).

Another feature of Hymenolepis diminuta is that it undergoes a diurnal migration within the rat intestine, which has been correlated with the feeding regime of the host. Essentially the worm moves along with the ingested food, along the length of the small intestine, and then migrates back to the top, ready for its next meal (Cornford 1990).

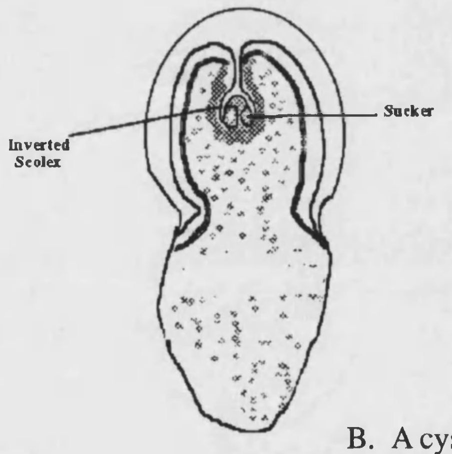
A further feature of H.diminuta is that the cysticercoids interfere with the endocrinology of their hosts, Tenebrio molitor. They affect vitallogenesis in female beetles, reduce patency, delay the second peak of egg laying and reduce the viability of the eggs. It is thought that these effects are caused either by reduced levels of circulating Juvenile hormone or that there is decreased tissue sensitivity to it (Hurd & Weaver 1987).

1.2.1.1 Structural Aspects of H.diminuta

The oncosphere of H.diminuta consists of several layers: an outer shell, an outer envelope, an inner envelope, embryophore, and the oncosphere. See Figure 13. The outer shell is formed by deposits laid down against the capsule and is composed of proteins and polysaccharides. The outer envelope is a syncytial cytoplasmic layer and has heavy deposits of lipid and phospholipid and disintegrates once the shell has formed. The inner envelope is composed of several cytoplasmic layers



A. An oncosphere



B. A cysticercoid

Figure 13. Diagram Of The Oncosphere And The Cysticercoid

Diagrammatic representation of the oncosphere and cysticercoid of Hymenolepis diminuta. A. Oncosphere. B. Cysticercoid.

and surrounds the embryo. The embryophore is a hardened layer secreted within the inner envelope. The oncosphere in the centre contains keratin hooks and a penetration gland (Pence 1970; Rybicka 1972).

The cysticercoid of H.diminuta is also shown in Figure 13. The cysticercoid consists primarily of an inverted scolex that is protected by the outer cyst wall of the cysticercoid. On stimulation in the definitive host the scolex everts to attach to the host gut wall (Smyth 1976).

Calcareous corpuscles are present in the intermediate stages of most cestodes e.g. cysticercoids of H.microstoma. They contain large quantities of inorganic substances which form a series of concentric rings. The corpuscles range in size from 10-34μm in diameter. Generally they are found throughout the parenchyma being composed of a mixture of calcium phosphate, calcium carbonate, magnesium ions, proteins, mucopolysaccharides, lipids and alkaline phosphatase (Lumsden & Hildreth 1983).

The calcareous corpuscles of H.microstoma are largely found in the cellular cortex. In their early stages of formation the cells where calcareous salts are being deposited appear vacuolated and appear to have no nucleus. The calcareous salts precipitate and accumulate along the myofibrils usually at the centre or edge of these putative cells. It is thought that the corpuscles may help to protect the intermediate stage during its passage through the acidic stomach of the definitive host, because at 168 hours post infection the calcareous corpuscles disappear. Alternatively the calcium salts may be involved in organisation and reorganisation processes in the developing larvae (Chowdhury & DeRyke 1977).

The structure of the adult proglottis is shown in Figure 14. It contains 3 testes, ovaries and uterus which are surrounded by parenchyma. Most of the worm is syncitial. The whole proglottis is protected by the tegument, below which lie the muscles, nerves and the excretory canals. Figure 15. The tegument is composed of a rapidly regenerated microthrix layer that is punctuated with the occasional sensory papillae. Immediately below the microthrix lies the distal cytoplasm, which is served by a network of tegumentary cytons. These have long internuncial processes that extend from below the basal lamella to the tegument. Cellular material is transported through these processes between the cell body and the distal cytoplasm. The latter contains a large number of vesicles, granules and mitochondria (Lumsden & Hildreth 1983).

Below the basal lamella lie two predominant forms of muscle, longitudinal muscle and circular muscle. Two other forms of muscle fibres are seen throughout the stobila these are the longitudinal medulary fibres and the cortical transverse fibres. Cestode muscle is smooth consisting of two myofibrils one thick with a diameter 100Å (10nm) and one thin with a diameter of 50Å (5nm). The muscle fibres contain 'dense bodies' associated with the sarcolemma which are thought to be fragmented Z-discs but they lack transverse-tubules (Lumsden & Byram III 1967; Lumsden & Hildreth 1983). A muscle cell has two components, the contractile myofibril and the myocyton. The myocyton contains the nucleus and is usually packed with glycogen together with free ribosomes, rough endoplasmic reticulum, an inconspicuous Golgi, the occasional lipid droplet and very large mitochondria. They are frequently some distance from the myofibrils and are connected to the fibres by tendrilled cytoplasmic processes. Often myofibres are connected to more than one myocyton. The peripheral cytoplasm of the myofibrils contains microtubules, mitochondria and glycogen (Lumsden & Hildreth 1983). In many cases there are also sarcoplasmic extentions from the muscles to the nerves (Webb & Davey 1975 & 1976).

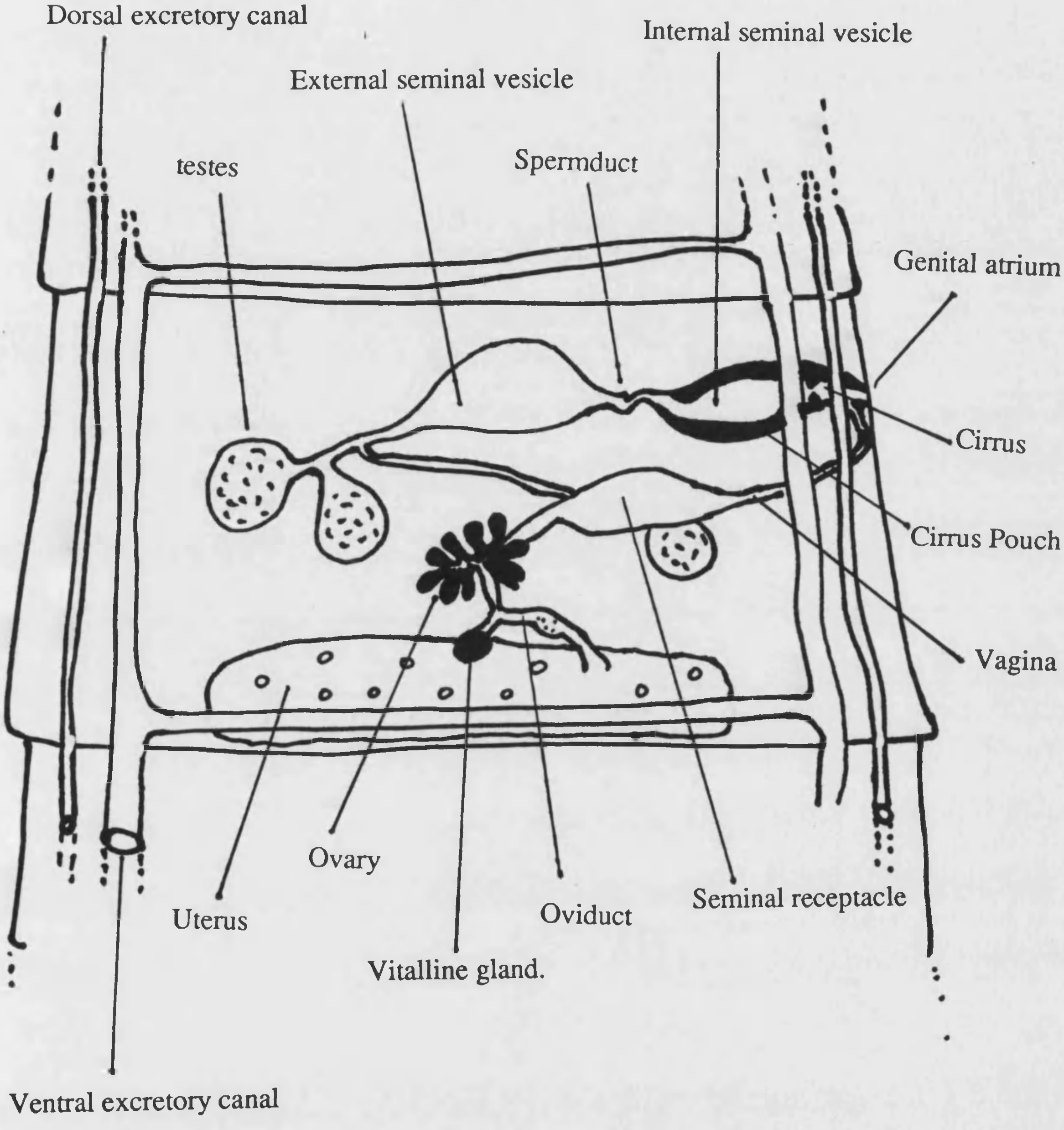


Figure 14. The Structure Of A Proglottid Of <u>H.diminuta</u>

A mature proglottid contains fully developed reproductive organs except for the uterus which does not develop fully until the oncospheres are produced.

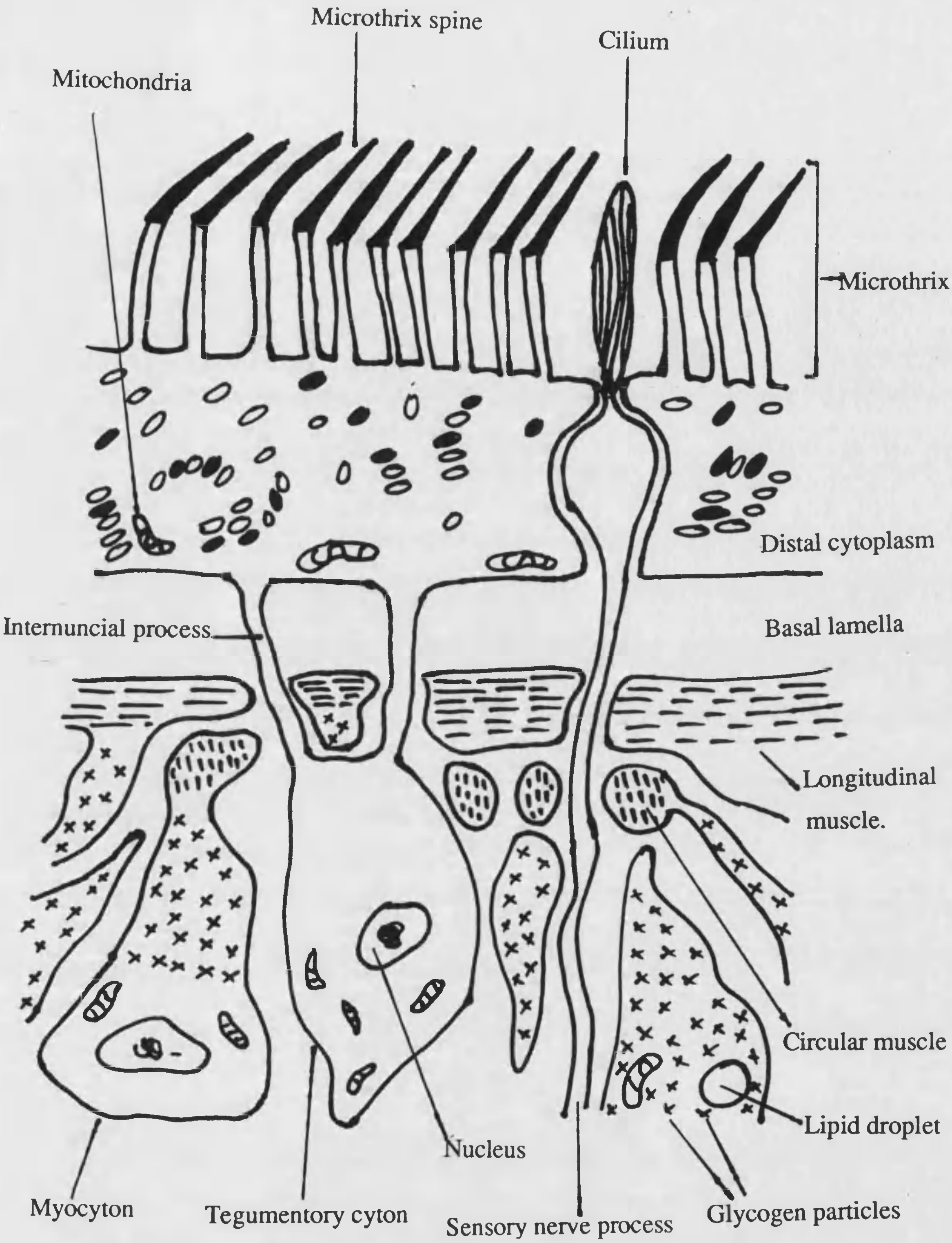


Figure 15. Structure Of The Tegument In <u>H.diminuta</u>

This is a diagrammatic representation of the structure of the tegument which is composed of the outer microthrix, the distal cytoplasm, the basal lamella and the underlying structures.

H.diminuta possesses a 'central nervous system' however the nervous tissue lacks a definite capsule or sheath and there are no glial cells. See Figure 16. There is a single pair of bilateral rostellar ganglia that handle both sensory input and motor functions, which are joined by a transverse commissure. Intense aceytlcholinesterase activity has been found in the rostellum however the only other neurotransmitter found, in H.diminuta, is 5'hydroxytryptamine (Wilson & Schiller 1969; Webb & Davey 1975 & 1976; Specian et al. 1979; Specian, & Lumsden 1980).

There are also 10 longitudinal nerve 'cords' which extend posteriorly through the strobila, with five on each side of the tapeworm. Of these two form major lateral nerve fibres, four form two minor pairs of lateral nerve fibres, four form a pair of medio-dorsal and a pair of medio-ventral minor nerve fibres. There are a series of ring commissures which link up the nerve fibres in each proglottid forming small ganglia. These have numerous nerve fibres that extend into the surrounding musculature and receives dendritic endings from sensory papillae. In the gravid proglottids however, most of the nervous tissue degenerates leaving only the major and minor lateral nerve fibres (Lumsden & Specian 1980; Lumsden & Hildreth 1983).

The protonephridial system consists of three major elements: large tubular canals, flame cells, and small tubules that are involved in excretion and the regulation of body fluid composition. There are four large canals, which extend the entire length of the tapeworm and end at the excretory pore in the terminal proglottid. If the terminal proglottid is shed, by apolysis, then the 4 canals independently open instead of joining to form a terminal pore. The flame cells, also referred to as protonephridia, are found throughout the strobila and scolex. They are believed to act as filters for the entrance of extracellular material and the propulsion of fluid through the

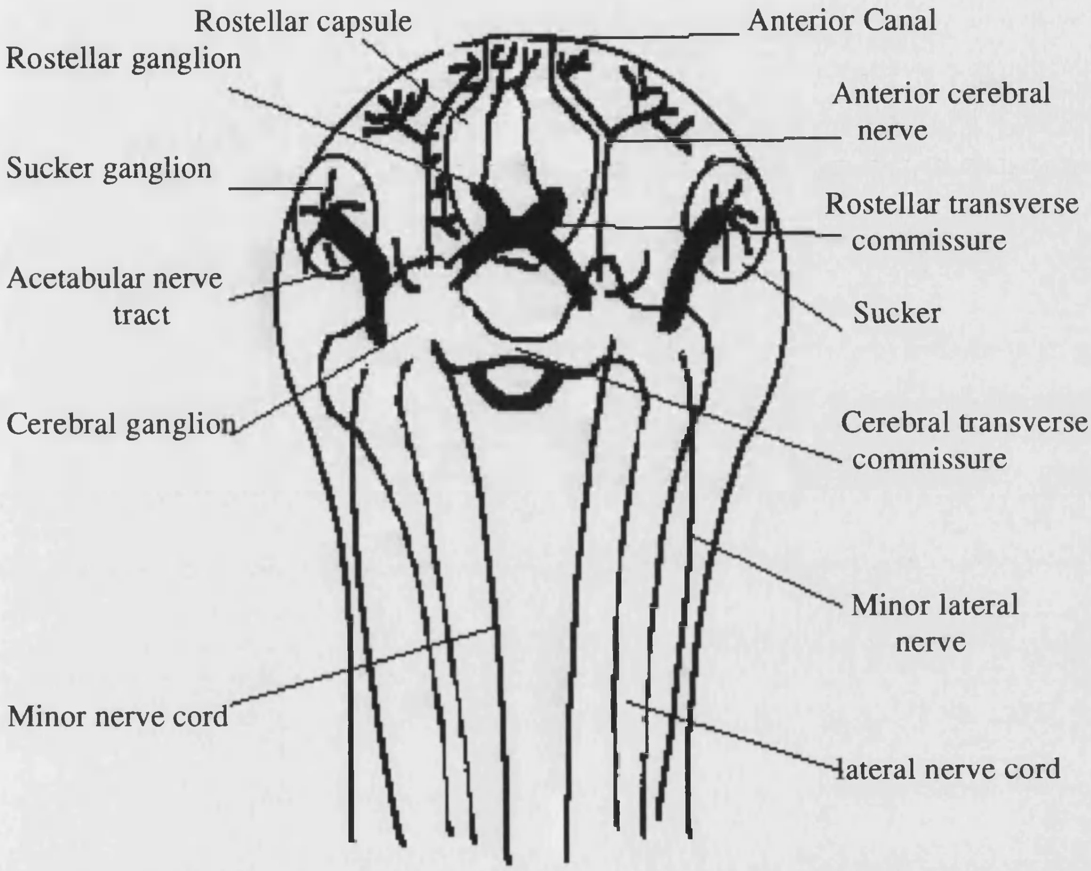


Figure 16. Diagram Of The Nervous System Within The Scolex

There are two major lateral nerve cords which extend along the body of H.diminuta. These arise from a central ganglion from which several other nerve cords and commissures extend (Lumsden & Specian 1980).

excretory system as they are composed of numerous cilia/flagella. The flagella have the conventional 9+2 microtubular axoneme and there are between 30-100 in a flame cell. Lastly the smaller tubules serve to connect the flame cells with the large excretory canals. The walls of the tubules and collecting canals are all syncytial and are covered with microvilli (Lumsden & Specian 1980; Lumsden & Hildreth 1983).

Phosphatases have been found associated with the ducts of the excretory system and appear to be involved in the active transport of substances across the wall of the excretory ducts. Mg^{2+} ATPase activity has also been found associated with these ducts and is thought to be involved in the movement of ions across the epithelial wall of the duct (Parshad & Guraya 1977). Both of these are thought to enable the worm to respond to ionic and osmotic stresses (Lumsden & Specian 1980; Lumsden & Hildreth 1983).

Distributed throughout the medulla are unicellular gland cells. They are frequently some distance away from the recognizable nerve tracts. These gland cells contain opaque granules and were originally thought to be neurosecretory. However, later work has shown them to be non-neural (Webb & Davey 1975 & 1976; Specian et al. 1979).

1.2.1.2 Reproduction in H.diminuta

Spermatogenesis proceeds from a primary spermatogonium which gives rise to 8 quaternary spermatogonia. These are arranged in a rosette and give rise to 16 primary spermatocytes. The spermatocytes then divide into 32 secondary spermatocytes which further divide into 64 spermatid nuclei which are arranged to form a syncitial cluster. Mature spermatozoa are 250-300μm long, with a broad head and a long cylindrical tail. There are two major differences between spermatogenesis in cestodes and that in higher eukaryotes. The first is that there are four major mitotic divisions proceeded by two meiotic divisions that give rise to 64 spermatid. The second is that the axoneme has a 9+1 arrangement. Spermatogenesis is protanderic, i.e. it is completed before oogenesis, there are three testes, which are functional for 4 days and spermatogenesis takes place in approximately 48 hours (Kelsoe, Ubelaker & Allison 1977; Lumsden & Specian 1980). Mature spermatozoa are passed down the sperm ducts into an 'external seminal vesicle' where they are stored until being passed through the cirrus, the copulatory organ, and into the vagina where they migrate to the seminal vesicle. This may occur either within one proglottid, or between worms.

Oogenesis takes place within the ovary and is the same as in higher eukaryotes. The ova mature on passing into the oviduct where fertilization will take place. The fertilised ovum forms a zygote, which fuses with a vitelline cell that has been secreted from the vitalline gland, which provides precursor material for the capsule and nutritional materials for the developing embryo. After fusion, the zygote migrates through the ovovitalline canal into the ootype (primary uterus) where numerous different secretions are made. The fully formed egg then leaves the ootype and travels to the uterus where the embryo develops to maturity (Lumsden & Specian 1980).

1.2.2 Biochemistry of H.diminuta

1.2.2.1 The Biochemistry of the Tegument

The tegument is the absorptive/excretory surface of the worm, and contains a large number of specific transporters. It also contains enzymes that are both produced by the cestode and absorbed from the host (Pappas, Uglem & Read 1973; Insler 1989). Both phosphodiesterase and ATPase activities have been found in the tegument of H.diminuta and are stimulated by divalent cations such as Ca^{2+} and Mg^{2+} (Gamble & Pappas 1981; Pappas 1981). There is also a tegumental alkaline phosphatase and a 5'nucleotidase (Pappas 1984). Significant differences in the levels of these enzymes occur within the tegument as the proglottids mature. The oldest proglottids having the lowest levels (Pappas, Narcisi, Rentko 1983). Glutathione transferase activity has also been found along the length of H.diminuta. Glutathione (GSH) transferases are involved in detoxifying xenobiotics and endogenously derived toxic compounds. It is thought that they may be acting as binding or transport proteins for ligands such as bile acids and haem (Brophy & Barrett 1990).

1.2.2.2 Carbohydrate metabolism

Tapeworms may assimilate up to 50% of the host ingested carbohydrate. Glucose uptake is through a stereospecific active transporter in the tegument that up regulates, in response to substrate depletion, within 24 hours (Cornford 1990). Glucose is temporarily incorporated into intermediate molecular weight glycogen, before incorporation into high or low molecular weight glycogen, which results in a constant assembly/dissassembly of glycogen stores (Roberts, Bueding & Orrell 1972; Roberts 1983).

Glycogen forms' 8-50% of the dry weight of H.diminuta and is present in two forms, α and β particles. The β particles are ≈20nm and the α particles, which tend to form glycogen rosettes, are 60-200nm. The molecular weight of glycogen in H.diminuta is unusual in that ≈30% of the total glycogen is very high molecular weight, average 900 million and ≈60-70% has an M*r* of 25-60 million (Orrell, Bueding & Colucci 1966; Colucci et al. 1966; Roberts, Bueding & Orrell 1972). The level of glycogen within the tapeworm depends on the state of the host and the stage of development of tapeworm. If the definitive host is fasting, then the glycogen stores rapidly become depleted, losing 60% within 24 hours (Lumsden 1966; Lumsden & Specian 1980; Roberts 1983). During the pre-patent period the glycogen concentration in the entire worm undergoes a massive increase. It also increases in the developing oncospheres during embryonation and concentrates around the developing muscles and the penetration glands (Dendinger & Roberts 1977).

In the adult worm, glycogen is found throughout the parenchyma but very little is found in the tegument, ovaries, testes or the oocytes (Daugherty & Taylor 1956; Fairbairn et al. 1961; Lumsden 1966; Moczon 1977; Lumsden & Specian 1980, Roberts 1983). H.diminuta possesses both glucose-6-phosphate independent (Type I) and glucose-6-phosphate dependent (Type D) glycogen synthase (UDP-glucose: α-1,4-glucan α-4-glucosyl transferase). The D-form is dephosphorylated by a protein phosphatase so converting it into the I-form, and the I-form is phosphorylated by a protein kinase (Dendinger & Roberts 1977; Moczon 1977). Glycogen phosphorylase is also present and exists in both active and inactive forms but it requires pyridoxal phosphate as a coenzyme (Read 1951). There is also histochemical evidence for phosphorylase *a* phosphatase, phosphorylase *b* kinase, cAMP dependent protein kinase and 1,4-α glucan branching enzyme (Moczon 1977 & 1977). Consequently it is believed that cestodes utilise the Embden-Meyerhof pathway for glycolysis as hexokinase, pyruvate kinase, lactate dehydrogenase, phosphoenolpyruvate carboxykinase and malate dehydrogenase have all been found in tapeworm tissue (Prescott & Campbell 1965; Bueding & Saz 1968; Burke et al. 1972; Kommunieki, Roberts 1977; Moon et al. 1977).

Tapeworms have a predominantly anaerobic energy metabolism consequently the tricarboxylicacid cycle is absent or of doubtful physiological significance. The major end-products of carbohydrate metabolism in cestodes are succinate, acetate (mitochondrial), lactate (cytosol), alanine and propionate (not demonstrated in H.diminuta) (Roberts 1983, Smyth 1976). It has been postulated that succinate and lactic acid production are regulated by the pH of the external milieu of the worm. If the medium is acidic, as in the duodenum, then the succinate pathway would be favoured because succinate is twice as effective at removing H^+ than lactate and so would be able to combat the acid stress. However, further down the gut the medium becomes less acidic and so the lactate pathway would be favoured. Although this is an attractive hypothesis it has been shown that the quantity of succinate produced would be insufficient to account for sufficient H^+ removal. But the combined production of lactate and acetate would be sufficient to prevent tissue acidification (Roberts 1983).

The mitochondria of H.diminuta have been shown to oxidise several substrates, e.g. α-glycerophosphate, succinate and pyruvate. Also, succinate dehydrogenase, cytochrome oxidase and peroxidase activity have all been demonstrated. The presence of peroxidase suggests that it is involved in the removal of toxic hydrogen peroxide formed during substrate oxidation. The mitochondria also contain non-haem iron (Cheah 1983).

1.2.2.3 Amino Acids

The transamination capabilities of H.diminuta appear to be quite limited. This suggests that it does not rely heavily on synthesis to satisfy amino acid requirements or catabolize amino acids to any significant degree for energy production. Both alanine aminotransferase and aspartate aminotransferase are present. Alanine can act as an amino donor to α–ketogluterate whereas aspartate and asparagine can act as donors to pyruvate and α-ketogluterate. Glutamate can act as a donor to pyruvate. Alanine accounts for 50% of the amino acids excreted (Harris 1983).

1.2.2.4 Lipid metabolism

In H.diminuta 5.8% of wet weight, 30.9% dry weight is lipid of which 70% is fatty acids, 24% is phospholipid and 10% is cholesterol and unsaponifiable lipids. 30% of an oncospheres dry weight is lipid. However, the quantity of lipid in the worm depends on the amount of lipid in the host diet, the age of the worm, density of infection and the host species (Hedrick 1957; Fairbairn et al. 1961; King & Lumsden 1969; Barrett 1983).

It is generally found in the parenchyma where it forms lipid drops in the myocytons and is concentrated around the excretory canals. Lipid drops also form in calcareous corpuscles and the reproductive organs (Hedrick 1957; Fairbairn et al. 1961; King & Lumsden 1969; Barrett 1983). The lipid content varies along the tapeworm, with the lowest content in the germinative region and the highest in the gravid proglottides. It has been suggested that the lipid in the gravid proglottides is to attract a host to ingest the eggs (Barrett 1983).

The lipids found are free fatty acids, monoacylglycerols, diacylglycerols, triacylglycerols, sterols and sterol esters and hydrocarbons. In most eukaryotes the major fatty acids are C_{16}-C_{18}, in H.diminuta C_{18} accounts for 70-80% of the lipid, of which linoleic acid $C_{18:2}$ is dominant. Between 80-90% of these C_{18}'s are actually unsaturated. Overall 70-80% of cestodal lipid is unsaturated (King & Lumsden 1969; Barrett 1983).

The triacylglycerols in H.diminuta are esterified at the C_2 position and show a high degree of stereospecificty with 92% being unsaturated. Mono- and di- acylglycerals are present in cestodes but form less than 1% of the lipid pool, unlike the triglycerols that account for 60% (Fairbairn et al. 1961; Barrett 1983). H.diminuta can manufacture triacylglycerols using the phosphatidic acid/diaclyglycerol pathway. For example, lysolecithin, which cestodes readily absorb, can be hydrolysed to lysophosphatidic acid, which can then be used to synthesise triacylglycerides. Phosphatidylserine is synthesised by the following mechanism:

Phosphatidic acid + CTP $\rightarrow$ CDP-diacylglycerol + pyrophosphate

CDP-diacylglycerol + serine $\Rightarrow$ CDP-diacylglycerol + CMP

Phosphatidylinositol synthesis is largely by the same reaction except that inositol is substituted for serine. Phosphatidylcholine and phosphotidylethanolamine are the major components of the phospholipids whilst phosphatidylserine and phosphatidylinositol are only a minor component, 5-10% (Barrett 1983).

Most cestode tissue has been found to contain sphingolipids, in particular sphingomyelins. Glucose cerebrosides (glycosphingolipids) have also been found in typically being $C_{24:0}$ and $C_{24:1}$. In mammal's glucose cerebrosides are characteristic of non-nervous tissue whilst galactose cerebrosides are characteristic of nervous tissue (Fairbairn et al. 1961; Barrett 1983). It is thought that sphingolipid synthesis is the same as that in higher eukaryotes, i.e.:

Palmitoyl-CoA + serine → 3'dehydrosphinganine → sphinganine ⇒ sphingosine

Phospholipids have been found distributed throughout the parenchyma and localised to the osmoregulatory canals, the walls of the seminal vesicles, ejaculatory duct, vagina and the seminal receptical (Hedrick 1957). Uptake of hexoses such as glucose, galactose and mannitol appear to inhibit incorporation of phosphate into phospholipids (Ip & Khan 1989). Cardiolipin, which is found on the inner mitochondrial membrane, has been detected in small quantities with plasminogens (Fairbairn et al. 1961; Barrett 1983).

Cestodes cannot synthesise long chain fatty acids, neither do they appear to be able to desaturate preformed fatty acids. However, H.diminuta can lengthen $C_{16:0}$ and $C_{18:0}$ to C_{20}, C_{22}, C_{24} and C_{26} by the addition of acetyl CoenzymeA.

The major steroid found in cestodes is cholesterol. Cestodes are incapable of synthesising steroids, which is probably because steroid synthesis has an absolute requirement for oxygen. However they are able to manufacture isopreniods from acetate and mevalonate such as 2-trans and 6-trans farnesol (a terpene). Farnesol is a precursor of higher prenoids, and is also a juvenile hormone mimic (Fioravanti et al. 1989).

There is no evidence for a β-oxidation path in cestodes, probably because it requires an aerobic environment. However several enzymes belonging to this pathway have been found that display activity *in vitro*. They include Acyl-CoA synthetase, acyl-CoA dehydrogenase, and acetyl-CoA acyltransferase. However, cestodes possess no known methods of catabolizing lipids (Barrett 1983).

1.3 REASONS FOR STUDYING CALMODULIN IN CESTODES

The main reason for studying calmodulin in the eucestoda is that so very little is known about their cellular biochemical processes.

A study on the effects of trifluroperazine (TFP), on H.diminuta revealed that TFP causes severe damage to the tegumental structure. TFP is a member of the hydrophobic antipsychotics that inhibits calmodulin action. It was thought that calmodulin or one of its target proteins may therefore be involved in the mechanism of TFP's action. In this same study biologically active calmodulin was successfully isolated and shown to have a molecular weight of 16.9kD (rod gel electrophoresis). It activated bovine phosphodiesterase in a Ca^{2+} dependent fashion. Also in this study an alkaline ATPase, thought to be a Ca^{2+} ATPase was isolated from the cestode, with an optimum activity at pH7.5. It was thought that this may be regulated by calmodulin, and therefore affected by TFP (Hipkiss 1986).

Also, actions of antihelminthics appear to have effects on cellular processes that are regulated by calcium and calmodulin in higher eukaryotes. An investigation is required to establish if this is true. If calmodulin, or its target proteins, are involved in the mechanism of action of these drugs, then new drugs could be designed which would be more efficient. A problem with present antihelminthics is that they exert their effects on the adult worm without affecting the eggs that it contains. If a drug was designed that could target calmodulin then it may be possible to affect both the eggs and the adult worm.

From an evolutionary stance it would be interesting to obtain the amino acid sequence for calmodulin. Comparisons could then be made with other known calmodulin sequences and an evolutionary tree developed. It would also be of interest to establish the number of genes encoding calmodulin.

This project was designed to further investigate calmodulin within the tapeworm H.diminuta. An attempt was made to isolate the calmodulin gene and sequence it using molecular biology. However, technical problems were encountered in obtaining high quality DNA that led to this approach being abandoned. Instead calmodulin was investigated using protein chemistry.

A novel method for isolating calmodulin from H.diminuta was developed. The isolated calmodulin was then analysed for its antigenicity and its biological activity.

The distribution of calmodulin within the whole worm was also determined using immunocytochemistry. Finally, preliminary investigations into the calmodulin binding proteins were undertaken.

2 MATERIALS AND METHODS

Three main approaches were used to investigate calmodulin in the cestode, H.diminuta. The first was a molecular biological approach to isolate the calmodulin gene. The second was to utilise protein chemistry to isolate the calmodulin protein and to characterise it. The third was to use immunocytochemistry to evaluate the distribution of calmodulin in the whole worm. In this chapter the methods used in each of these approaches is described.

Unless otherwise stated all reagents were from either Sigma Chemical Company Ltd. or BDH and were of Analar Grade.

2.1 Maintenance of H. diminuta

Adult tapeworms were maintained in male Wistar rats that were orally infected at a weight of 250-300g. The adult worms (patent) were extracted from the small intestine by flushing it with ice cold saline and then temporarily stored in saline. The terminal proglottides, being gravid, were used for infection of the intermediary host, which was Tenebrio molitor. Before infection with macerated proglottides the beetles were starved for at least 24 hours. After 24 hour's exposure to the tapeworm tissue, containing oncospheres, the beetles were returned to their normal diet of apples.

Beetles were maintained at room temperature. After 20 days or more they were sacrificed and the cysticercoids extracted. The cysticercoids were then orally dosed to the rats.

2.2 Molecular Biology

2.2.1 DNA Extraction

Several methods were used, all of which will be described here. However, difficulty was found in obtaining high quality DNA that was >23kb as it was either heavily contaminated with glycogen or it was badly sheared.

Materials

The materials listed are for all the DNA isolation procedures described.

- Tris(hydroxymethyl)methylamine (tris) 1M pH7.4, 8.0, 9.0 (autoclaved)
- 0.5M EDTA (autoclaved)
- 4.5M Sodium acetate pH 6.5 (autoclaved)
- 5M NaCl (autoclaved)
- 10% (w/v) Sodium dodecyl sulphate (SDS)
- 5M NaOH
- Ethanol 95% & 70% (v/v)
- Iso-amyl alcohol
- Redistilled phenol (Rathburn Chemicals Ltd.)
- Ribonuclease A (Sigma R-5000) 10mg/ml solution. This was boiled for 10 minutes prior to use to remove any DNAase that may be present.
- Protein Kinase *aus tritiraclun album* 10mg/ml solution (Boehringer Mannheim GmbH 745-723).
- TEN9 Buffer: 50mM Tris-HCl pH9, 100mM EDTA, 200mM NaCl. *Made up into 20ml aliquots.*
- TE Buffer: 10mM Tris pH 8, 1mM EDTA pH 8.
- CTAB Buffer: 2% (w/v) cetyl trimethyl ammonium bromide (CTAB), 0.1M Tris-HCl pH 8, 2mM EDTA, 1.4M NaCl, 1% (w/v) polyvinylpyrolidone.
- 5% (w/v) CTAB
- Chloroform/iso-amyl alcohol 24:1.
- Precipitation Buffer: 1% (w/v) CTAB, 50mM Tris-HCl pH 8, 10mM EDTA.

- Dialysis Buffer: 50mM Tris-HCl pH8.0, 10mM EDTA pH 8.0.
- Molecular Biosystems Inc. Kit for DNA extraction. Included Specimen dilution reagent, lysing reagent, wash reagent, elution reagent and a column.
- Saponin Buffer: 1% (w/v) Saponin, 0.5% (v/v) Triton, 100μg/ml of RNAase in TEN9 buffer.
- Nuclei Buffer: 12mM Tris-HCl pH7.5, 0.32M Sucrose, 3mM $MgCl_2$ 5mM β-mercaptoethanol.

Methods

2.2.1.1 Method 1: Proteinase K And Phenol Extraction

This method is based on that proposed by Herrmann and Frischauf (1987).

1. 2.5g wet weight tapeworm tissue was cut into $1cm^2$ pieces and frozen with liquid nitrogen and ground into a powder with a pestle and mortar.
2. The powder was removed and placed in a sterile conical flask containing 20ml of TEN9 buffer. This was mixed until the powder had dissolved.
3. DNAase free RNAase A was added to give a final concentration of 100μg/ml. The flask was then placed on a shaking platform, for 10 minutes, at room temperature.
4. To this mixture 1ml of 20% (w/v) SDS was added. It was mixed for a further 10 minutes. This is to aid the dissociation of proteins from the nucleic acids. SDS also has a mild inhibitory effect on endogenous nucleases.
5. Then 1ml of proteinase K (10mg/ml) was added and the flask incubated overnight on a shaking waterbath at 37°C.
6. 20ml of Tris-treated phenol pH 8 was then added, and the flask incubated at room temperature, for 3 hours on a shaking platform.
7. The contents of the flask were then transferred into 50ml sterile Falcon tubes, which were centrifuged at 3000rpm for ≈15 minutes, in a benchtop centrifuge.

8. As a result of this spin two phases were produced, an aqueous phase containing nucleic acid and a phenolic phase containing the protein. The phenolic phase was removed, and the aqueous phase retained. If necessary, steps' 6-8 were repeated until the aqueous phase was clear.

9. To remove any residual contaminating protein the clear aqueous phase was then centrifuged at 5000g in a Corex tube for 20 minutes, at 25°C, using a Sorval centrifuge.

10. The supernatant was removed. There were then two possible methods for obtaining the DNA either A or B.

<u>A</u>

The supernatant was dialysed against four changes of TE or dialysis buffer at 4°C until the absorbance of the dialysate was ≤ 0.05 at 280nm. It was then brought to 0.3M sodium acetate, by the addition of stock 4.5M sodium acetate pH 6.5. To this mixture 0.8 volumes of 2-iso-propanol were added. It was then gently mixed by inversion of the tube. The precipitated DNA could then be spooled out with a pipette and resuspended in TE buffer.

<u>B</u>

The supernatant was added, with gentle mixing, to an equal volume of chloroform and left to stand for 10 minutes at room temperature. This mixture was then centrifuged at 10,000g for 10 minutes, at 4°C. The aqueous phase was removed and brought to 0.3M sodium acetate using the 4.5M stock pH 6.5. To this mixture 2 volumes of 95% (w/v) ethanol (pre-chilled at -20°C) were added and gently mixed. An attempt to spool out the DNA was made if this failed then the mixture was centrifuged at 10,000g for 15 minutes, at 4°C.

The supernatant was discarded and the pellet resuspended in 70% (w/v) ethanol (pre-chilled at -20°C) prior to being dried with a Speed-Vac Concentrator (Savant Instruments Inc.). The resulting pellet was resuspended in 200µl of TE buffer.

2.2.1.2 Method 2: CTAB

In an attempt to overcome the glycogen contamination of the DNA the following method was used because it is very efficient at removing polysaccharides

1. 2.5g of powdered frozen tissue (as per method 1, section 2.2.1.1.) was added to 10ml of preheated CTAB buffer (65°C) in a Falcon tube. This was then incubated for 15 minutes at 56°C in a shaking waterbath until the powdered tissue had dissolved.
2. To this mixture, 10ml of chloroform/ iso-amyl alcohol (24:1) was added. The tube was then inverted several times to gently mix the contents before being centrifuged at 10,000g for 10 minutes, at 20°C.
3. The aqueous phase was removed to a fresh Falcon tube and 0.2 volumes of 5% CTAB were added and mixed. The chloroform layer was discarded.
4. Steps' 2-3 were repeated.
5. The final aqueous phase was transferred to a 30ml Corex tube and an equal volume of precipitation buffer added. The tube was sealed and gently mixed by inversion before being centrifuged at 3000g for 15 mins, at 20°C. The resulting supernatant was discarded.
6. The pellet was resuspended in 2ml of TE buffer containing 1M NaCl. If necessary this was aided by warming at 56°C for ≈10 mins.

7. The sample was then placed on ice and 2 volumes of pre-chilled (-20°C) 95% (v/v) ethanol was added. This was mixed, and the DNA spooled out if possible. If the DNA could not be spooled out then the sample was centrifuged at 10,000g for 10 mins, at 4°C and the supernatant discarded. The pellet was resuspended in 70% (v/v) ethanol (pre-chilled -20°) and dried with the Speed Vac.
8. The resulting DNA pellet was resuspended in 0.2ml TE buffer pH7.5 at 4°C.

2.2.1.3 Method 3: Molecular Biosystems Inc. Kit.

1. 3g of powdered frozen tapeworm tissue was suspended in 6ml of "specimen dilution reagent". To this 600µl of 20mg/ml proteinase K was added and mixed thoroughly.
2. Then 6ml of "lysing reagent" was added and the mixture incubated for 30 mins in a shaking waterbath at 60° C.
3. At the same time the supplied column was prepared as per manufacturers' instructions. The sample was loaded onto the column and allowed to run through under gravity (maximum flow rate = 20 drop per minute). The column was then washed with 3ml "Wash reagent". Then 1ml of 'Elution reagent' was applied and 1ml eluent collected before a further 1ml elution reagent was applied. The 2ml of eluent containing the nucleotides were then pooled.

2.2.1.4 Method 4: Saponin/Triton/Phenol/Chloroform

An attempt was made to improve the extraction of DNA by using a combination of detergents. Saponin, a detergent, is known to strip the microthrix layer of tapeworms and this would remove a significant barrier to the reagents.

Tapeworms were mixed with TEN9 buffer containing 1% (w/v) saponin, 0.5% (v/v) triton and 100μg/ml RNAase. After a 30 minute incubation at 4°C, the mixture was centrifuged at 3000g. The supernatant containing the microthrix and the oncospheres was discarded. The pellet that contained relatively intact worms was rinsed with TEN9 buffer. These worms were then frozen with liquid nitrogen and made into a powder with a pestle and mortar. This was then used in either method 1 or 2 for DNA extraction (sections' 2.2.1.1 and 2.2.1.2.).

2.2.1.5 Method 5: Isolation of Nuclei/Proteinase K/Phenol Extraction.

It was hoped that if nuclei could be isolated then purer DNA could be extracted.

Small pieces of worm (1cm^3) were homogenised, with a Sorval blender, on ice in nuclei buffer. The homogenate was filtered through two layers of muslin cloth and the filtrate centrifuged for 10 mins at 500rpm. The supernatant was removed and centrifuged for 15 minutes at 5000rpm. The resulting pellet was resuspended in chilled nuclei buffer. This was then centrifuged at 5000rpm for 15 mins, the supernatant was discarded and the pellet resuspended in the required buffer for DNA extraction according to method 1, omitting the freezing stage (section 2.2.1.1.).

2.2.2 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to assess the size and quality of isolated DNA. It was also used to analyse digests of DNA and PCR products.

Materials

- Stock TBE Buffer x10: 0.89M Tris, 5.5% (w/v) Boric acid, 20mM EDTA.
- x1 TBE Running Buffer: Stock TBE diluted 1-10 with double distilled water.
- Loading Dye: 15% (w/v) Ficoll , 0.1% (w/v) Bromophenol blue, 0.1% (w/v) Xylene cyanole
- Agarose (Sigma electrophoresis grade)
- Stock Ethidium Bromide 1mg/ml in distilled water.
- Molecular Size Markers: Uncut and cut λ, and Hind III

Method

A 0.3% (w/v) agarose gel was used. This will separate DNA that is between 5-60kb.

1. A gel template was prepared using a gel cast and comb.
2. In a conical flask, 0.3g of agarose was added and mixed with 100ml x1 TBE. This was then microwaved until the agarose had dissolved to form a clear solution. Ethidium bromide was then added to give a final concentration of 0.5μg/ml using the stock ethidium bromide solution. This mixture was poured into the gel template and allowed to set at 4°C for ≈1hour. Once set the comb was removed and the gel placed in the gel tank, which was filled with x1 TBE.
3. The DNA sample was mixed with an equal volume of loading dye and loaded into the gel. The gel was run slowly at 30mA until the loading dye was ≈1cm from the

end of the gel. It was then viewed with a UV transilluminator and photographed with a Polaroid camera.

2.2.3 Southern Blots of Agarose Gels

Southern Blots were used to transfer DNA from agarose gels to nitro-cellulose membranes. Once on the membrane the DNA could be probed with radiolabelled oligonucleotides that were complementary to a specific sequence of a gene (Southern 1975).

2.2.3.1 Transfer of DNA from Gel to Genescreen™ Membrane

Materials

- Pre-Hybridization Solution: 1M NaCl, 1% (w/v) sodium dodecyl sulphate (SDS).
- Denaturing Solution: 0.5m NaOH, 1.5M NaCl
- Neutralising Solution: 0.5M Tris pH7.5, 1.5M NaCl.
- x20 SSC: 3M NaCl, 300mM Sodium citrate pH7, 4% (w/v) SDS.
- x10 SSC: 1.5M NaCl, 150mM Sodium citrate pH 7, 2% (w/v) SDS.
- x1 SSC: 0.15M NaCl, 15mM Sodium citrate pH 7, 0.2% (w/v) SDS.
- Genescreen Plus™ hybridization transfer membrane (DuPont)
- Whatmans 3MM Filter Paper
- Paper Towels
- Clingfilm
- Heavy Weight

Method

1. The agarose gel prepared according to section 2.2.2. was soaked in denaturing solution for 30 minutes to separate the strands of DNA. It was then transferred

from the denaturing solution to the neutralising solution where it was incubated for a further 30 minutes.

2. Simultaneously the GeneScreen™ paper was prepared. After the desired size of paper had been cut it was washed in double distilled water. To ensure the correct side was used for the transfer, it was noted which way the paper curled when wet. The inside of the curl was the side that was placed next to the DNA on the gel. Therefore, this was marked with a small cross in the corner using a pencil before being rinsed with x20 SSC.
3. The two chambers of a Southern tank were filled with x10 SSC. See Figure 16. The Whatman 3MM paper was wetted with x10 SSC and carefully laid across the bridge ensuring that each end extended into the chambers containing the buffer. Care was taken to ensure that no air bubbles were trapped underneath.
4. The agarose gel was placed onto the filter paper in the Southern tank, with the base of the gel being uppermost. This represents the base of the wells of the gel and contains the DNA. The Genescreen™ membrane was then carefully laid over the gel ensuring that there were no air bubbles present.
5. A piece of Whatman 3MM was cut to the size of the gel and placed over the Genescreen™. Clingfilm was then placed on either side of the gel to prevent short circuits and several layers of hand towels placed on top of the gel, Genescreen™ and Whatman 3MM paper. Finally a ≈2kg weight was placed on top of the hand towels and the blot left overnight at room temperature.

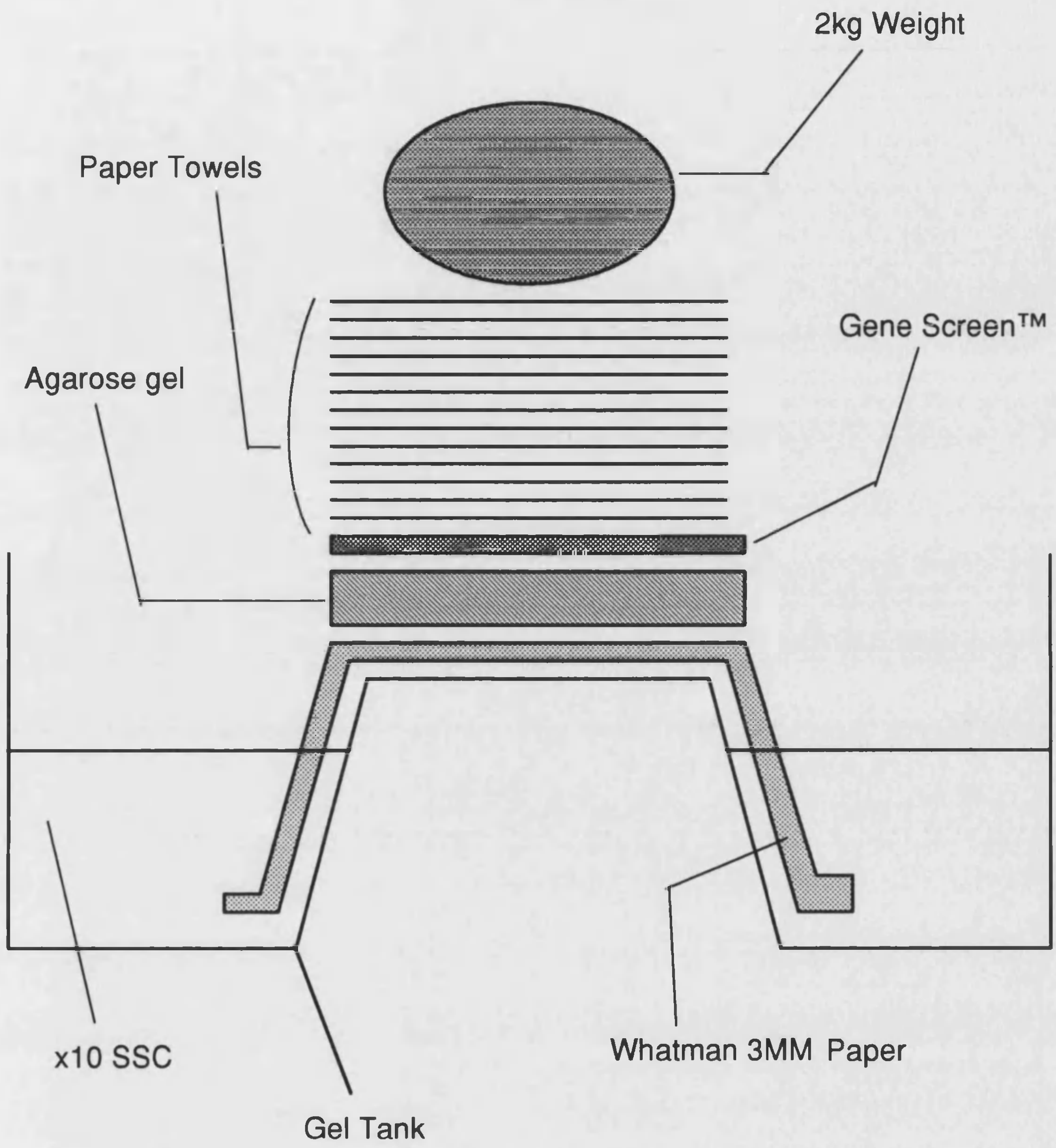


Figure 17. Diagram Showing the Apparatus for a Southern Blot

The two chambers of the gel tank were filled with x10 SSC. A wick of Whatmans 3MM paper was laid across the bridge of the tank, on top of this, was placed the gel, Genescreen™, paper towels and weight. These were then left overnight.

2.2.3.2 Probing Of Genescreen™ For Gene

Once the DNA was transferred to the GeneScreen™ it was probed with an oligonucleotide that was complementary to part of the calmodulin gene.

Materials

- x2 SSC: 0.3M NaCl, 30mM Sodium citrate, 0.4% (w/v) sodium dodecyl sulphate (SDS).
- Denaturing Solution: 0.5m NaOH, 1.5m NaCl
- Neutralising Solution: 0.5M Tris pH7.5, 1.5M NaCl.
- Polynucleotide Kinase (Northern Biological Laboratories [NBL])
- 10x Buffer (supplied with polynucleotide kinase)
- Pre-Hybridization Solution: 1M NaCl, 1% (w/v) SDS.
- Oligonucleotide Probe: 50 bases from the second exon of calmodulin based on known calmodulin sequences (Donated by Dr.Alshareif).
- AT-γ-^{32}P (Amersham PB218 supplied in ethanol:water 1:1 at 74MBq/ml equivalent to 2mCi/ml)
- 1mM ATP
- Sephadex G25 (Pharmacia)
- Siliconized Glass Wool (donated)
- 1ml Sterile Glass Syringe
- TE Buffer: 10mM Tris pH 8, 1mM EDTA pH 8.
- 0.5M EDTA
- Paper Towels
- Polythene Plastic Sheet
- X-Ray Film (Fuji RX-100)

Method

1. Some paper towels were pre-dampened with denaturing solution.
2. The weight and all the blotting papers were removed from the Southern Blot (section 2.2.3.1.), leaving the GeneScreen™ on top. The GeneScreen™ was carefully removed with tweezers and transferred to the pre-moistened paper towels and then onto some paper towels that had been wetted with neutralising

solution. Finally it was placed into a container with x2 SSC, where it was left for 10 minutes.

3. Sufficient plastic sheeting was cut to form an envelope for the Genescreen™. The Genescreen™ was then placed inside and the plastic sheet folded and heat sealed on three sides leaving one side open. 20-50 ml of pre-hybridization solution was then placed in the bag, which was sealed and incubated at 60°C for 6 hours on a rotator.

4. During this time the oligonucleotide probe was end-labelled with ^{32}P as follows:

   a) 10 pmols of oligonucleotide were placed in a siliconised eppendorf. To which 5μl NBL 10x buffer and 1μl pf polynucleotide kinase were added together with sufficient double glass distilled water to bring the reaction volume to 50μl The reaction was started by the addition of 50μCi of AT-γ-^{32}P and incubated at 37°C for 30 minutes. After 25 minutes had elapsed 10μl 1mM ATP was added to maintain the ATP concentration so that the maximal amount of ^{32}P could be incorporated onto the oligonucleotide. The reaction was stopped by the addition of 10μl of 0.5M EDTA.

   b) During this incubation period a G25 sephadex column was prepared. Sterile glass wool was placed in the base of the sterile 1ml glass syringe that was filled with pre-swollen G25 Sephadex. The column was placed inside a test tube so that the eluent could be collected and centrifuged at 2000rpm for 4 minutes. The column was then washed once with TE buffer and twice with 50μl of double glass distilled water and centrifuged at 2000rpm for 4 minutes each time.

c) After the labelling reaction was completed the mixture was loaded onto the prepared Sephadex column. An eppendorf was taped to the base of the syringe to collect the eluate. This was then placed into the test tube before being centrifuged at 2000rpm for 4 minutes. Any unreacted ATP would remain in the column. The labelled oligonucleotide was then ready to probe the GeneScreen™.

5. The pre-hybridization fluid was removed from the bag containing the GeneScreen™ and the labelled oligonucleotide, from 4c., added. The bag was resealed and incubated on a shaking waterbath overnight.

6. The mixture was removed from the bag and the GeneScreen™ placed in a container. It was then rinsed several times with x2 SSC before being left to soak for two hours in x2 SSC. After which time it was then wrapped in clingfilm and placed in an autoradiograph cassette with X-ray film placed over it. The cassette was then placed in a -80°C freezer and left to develop for two days.

2.2.4 Digestion of Isolated DNA

Digestion of DNA by restriction enzymes was necessary to determine if restriction sites were present on the isolated DNA. If present, then the DNA could be partially digested to yield suitable sized fragments of DNA, which could be inserted into a vector, for construction of a genomic library.

Materials

- Restriction Enzymes: EcoR1, BamH1, Hind III, Pst1 and Sau3A (NBL).
- Restriction Buffer supplied with restriction enzymes (NBL).
- Sample DNA from section 2.2.1.
- Eppendorfs (siliconized)

Method

A sample of the tapeworm DNA (10-40μg) was placed in a series of eppendorf tubes and sufficient double glass distilled water added to make the total reaction volume either 20μl or 100μl. To this, either 2μl or 10μl of restriction buffer was added. Finally, one, or a combination of two, restriction enzymes were added at 0.1 unit per 100μl. The reaction vessels were then incubated for 3 hours at 37°C.

Once the reaction was complete the samples were analysed by agarose gel electrophoresis using 0.6%-1% gels (0.6% separates 1-20kb DNA, 1% separates 0.5-7kb DNA). If suitable fragments had formed then the gel was Southern Blotted and probed with radiolabelled oligonucleotide, as previously described (Section 2.2.3.).

2.2.5 Amplification of DNA

Up to this point in the work only DNA of less than 23kb could be obtained and even then in very small quantities. Thus, it was felt that amplification of the calmodulin gene by the polymerase chain reaction (PCR) would prove a more effective route.

In PCR two synthetic oligonucleotides are used as primers for a series of reactions catalysed by DNA polymerase. The oligonucleotide primers are of different sequences but complementary to sequences that both lie on opposite strands of template DNA and flank the region of DNA to be amplified. The reaction involves a cycle where the template DNA is first denatured in the presence of excess primers and dNTP's at 94°C. Then the mixture is cooled to a temperature that enables the primers to anneal to the target sequence on the template DNA (determined empirically). Finally, the annealed primers are extended by the DNA polymerase at

72°C. The products of each cycle serve as templates for successive cycles and so further enhance the amplification procedure.

2.2.5.1 Synthesis of Primers for Polymerase Chain Reaction (PCR)

Two primers were needed one for the 5'end of the gene and a second for the 3' end.

<u>5' Primer</u>

A region at the start of the second exon, of the calmodulin gene, was selected as it contained an EcoR1 restriction site (See below).

```
                         EcoR1 site
                             ↓
5'-GAA-GAG-CAG-ATC-GCA-[GAA-TTC]-AAA-GAA-GCT-TTC-
   -glu--glu--gln--ile--ala--glu--phe--lys--glu--ala--phe-

   TCC-CTA-TTT-GAC-AAG-GAC-GGG-GAT-GGG-ACA-3'
   -ser--leu--phe--asp--lys--asp--gly--asp--gly--thr-
```

As this was to be used to probe the cestode genome, the codon usage of <u>Taenia solium</u> was reviewed so that the base usage could be biased to that used in cestodes (Campos et al. 1990). Consequently the following 42 base sequence was synthesized:

```
5'-GAG-GAG-CAG-ATT-GCA-GAA-TTC-AAG-GAC-GCT-
-TTC-TCC-CTC-TTC-GAC-AAG-GAT-GGT-GAT-GGC-ACC-3'
```

<u>3' Primer</u>

This was selected from the end of the fourth exon as it contained a HpaI restriction site. The sequence chosen is shown overleaf.

Hpa1

5'-ATG-ACT-AAC-CTC-GCT-GAG-AAG-TTA-ACC-GAC-GAG-GAG-GTG-3'

As it was the 3' end that was required the sequence was reversed, and then the complementary strand of this synthesised. This is shown below.

HpaI

3'-CAC-CTC-GTC-GGT-TAA-CTT-CTC-ACC-GAG-GTT-AGT-CAT→

However, it was found necessary to incorporate additional restriction sites that would facilitate ligation into the cloning vectors. So that, immediately preceding the above code the following was added:

HgiA1

3'-GAG-CAC-AAG-CTT→

HindIII Alu1

The two primers were synthesised on controlled pore glass columns supplied by Cruachem in an Applied Biosystems DNA synthesiser Model 381A by the phosphoramidite method (Beaucage & Caruthers 1981; Applied Biosystems 1987).

2.2.5.2 Post synthesis Preparation of Oligonucleotide Primers

Materials

- Concentrated Ammonia
- 1ml and 5ml Syringe
- Glass columns containing oligonucleotide probes from section 2.2.5.1.
- TE Buffer: 10mM Tris pH8, 1mM EDTA
- 95% (v/v) Ethanol
- 3M Sodium Acetate

Method

1. The column on which the oligonucleotide was synthesised was removed from the Applied Biosystems DNA synthesiser and on one end of the column a 1ml syringe containing concentrated ammonia was attached. To the other end an empty 5ml syringe was affixed. The ammonia was then drawn through the column, in volumes of ≈0.2ml every 20 minutes and collected in the 5ml syringe. This was transferred to a screw cap eppendorf, which was sealed and baked in an oven overnight at 55°C.
2. The solution containing the oligonucleotide was then dried down in the Speed-Vac and the resulting pellet resuspended in 1ml TE buffer.
3. To this was added, 100µl of sodium acetate and 3ml 95% (v/v) ethanol and the mixture placed at -20°C for several hours to allow the oligonucleotide to precipitate. It was then centrifuged at 10,000rpm for 20 minutes and the pellet resuspended in 1ml double glass distilled water.
4. The oligonucleotide solution was scanned from 350nm-240nm. A clear sharp peak should occur at ≈260nm, from this the optical density is recorded and the concentration of the oligonucleotide calculated knowing that 1 OD=20µg/ml oligonucleotide.

2.2.5.3 Size Determination of Oligonucleotides

The oligonucleotide primers from section 2.2.5.2. were analysed by performing polyacrylamide gel electrophoresis. This is to verify that the synthesis proceeded as planned (Wallace & Miyada 1987).

Materials

- T4 Polynucleotide Kinase (NBL)
- T4 Polynucleotide Kinase x10 Buffer (Supplied with manufacturers enzyme)
- AT-γ-^{32}P (Amersham PB218 in ethanol:water 1:1 at 2mCi/ml; >5000Ci/mmol)
- Sequagel Solutions (Flowgen National Diagnostics)
- Sample Buffer:- 98% (v/v) formamide, 0.15% (w/v) bromophenol blue, 0.15% (w/v) xylene cyanole
- Stock TBE Buffer x10:- 0.89M Tris, 5.5% (w/v) Boric acid, 50mM EDTA.
- Oligonucleotides from section 2.2.5.2
- X-Ray Film (Fuji RX-100)

Method

1. The oligonucleotides were 5' end-labelled as follows.

   10 µl (0.15pmol) AT-γ-^{32}P was placed in an eppendorf and dried down in a Speed-Vac. To this, 100ng of oligonucleotide primer was added together with 5µl of polynucleotide kinase x10 buffer. Sufficient double glass distilled water was then added to bring the final volume to 50µl. The mixture was then incubated at 37°C for 30 minutes.

2. Simultaneously a 20% acrylamide gel was prepared using the Sequagel solutions as per manufacturers' instructions. This mixture was then quickly poured into the gel apparatus, which consisted of two glass plates separated by a spacer of 3mm. Waterproof tape was used to seal the sides. Once poured a thin comb was inserted and the gel allowed to set. Then the tape was removed from the base of the plates, and the gel placed in the electrophoresis chamber. It was then pre-run for approximately 30 minutes with blank samples to remove any undesirable impurities that could affect the resolution.

3. After the pre-run, 10µl of each of the oligonucleotide primers was mixed with 1µl of sample buffer and heated for 2 minutes at 70°C before being loaded into the gel. The gel was run at ≈150V/cm for 30 minutes. The two dyes in the sample buffer act as size markers, xylene cyanole migrates to a distance equivalent to 30 bases, and bromophenol blue migrates a distance equivalent to 15 bases. Consequently the two oligonucleotide primers will run behind both of these dyes as they are 42 and 45 bases respectively.
4. The gel was then carefully removed from the plates, wrapped with clingfilm and placed in an autoradiograph cassette, with a piece of x-ray film placed on top. It was then stored at -80°C and after a suitable length of exposure the film was removed and developed.

2.2.5.4 PCR Amplification of DNA

Materials

- 20µM Stock Oligonucleotide Primers (from Section 2.2.5.2)
- Amplitaq™ (Perkin Elmer)
- Stock Amplitaq™ Reaction Buffer x10:- 100mM Tris HCl pH 8.3 and 500mM KCl.
- Sample DNA (from section 2.2.1).
- 1.25mM dNTP and 4mM dNTP (dATP, dTTP, dCTP, dGTP)
- Stock 1M $MgCl_2$.
- Replinase™ (NBL)
- Replinase x20 Stock Buffer:- 1M Tris-HCl pH9.0, 30mM $MgCl_2$, 400mM $(NH_4)_2$ SO_4
- Nen-Sorb Columns (NEN Research Products Division, DuPont)
- Buffer A:-10ml Tris-HCl, 14µl triethylamine
- 50% (v/v) Iso-Propanol
- Low Melting Point Agarose (Bethsada Research Labs)
- Ethidium Bromide 1mg/ml in distilled water.
- Methanol
- CL6B Sepharose Gel (Pharmacia)
- TE Buffer: 10mM Tris pH 8.0, 1mM EDTA pH 8.0.
- Mineral Oil (Sigma M-3516)

Method

A separate set of micropipettes were used to ensure no contamination with DNA from other sources.

1. Either of the following reactions were set up using two different manufacturers DNA polymerase enzyme.

   A. PCR Reaction Using AmpliTaq™

   To a set of three autoclaved 0.5ml eppendorfs 10μl x10 AmpliTaq reaction buffer, 16μl dNTP's, 50μl distilled water, 5μl of each primer, 10 μl of sample DNA (equivalent to 10ng/μl in the reaction vessel) and 1μl of AmpliTaq™ (equivalent to 5U/μl) were added.

   B. PCR Reaction Using Replinase

   To another set of three autoclaved eppendorfs the following was added 1μl x20 Replinase stock buffer, 1μl 4mM dNTP's, 1μl 20μM primers, 5-15μl sample DNA (equivalent to 10ng/μl in the reaction vessel), 0.5μl replinase™ and sufficient distilled water to bring the sample volume to 100μl.

   The samples from each reaction were then brought to either 10, 20 or 30mM $MgCl_2$ by the addition of 1-3μl of 1M $MgCl_2$. Then 100μl of light mineral oil was laid over the reaction mixture to prevent evaporation of the contents.

2. The eppendorfs were then placed in a Perkin Elmer or Techne PCR machine, which was programmed for 30 cycles. Each cycle consisted of 5 minutes denaturing at 94°C, followed by 2 minutes annealing at a lower temperature, and 3 minutes polymerisation at 72°C. The annealing temperatures tried were either 52°C, 42°C or 37°C. Once the programme was completed then the oil was

removed from the reaction vessels, by the addition of 50μl chloroform. This was mixed and centrifuged for 4 minutes in a microfuge and the aqueous layer carefully removed.

3. The PCR products were analysed by 1% (w/v) agarose gel electrophoresis. (As previously described in section 2.2.2. but using Low Melting Point Agarose so that the DNA could easily be recovered). The gel was viewed with a UV transilluminator and the band containing the PCR product cut out with a scalpel. The PCR product was recovered by the process of "freeze-squeezing" which is described below.

4. Freeze squeeze:-

   Siliconised glass wool was packed at the base of a 0.5ml eppendorf, which had a small hole pierced in its base. The cut band of the PCR product was then added and the eppendorf placed inside a larger 1ml eppendorf. A long aluminium foil 'wand' was prepared with a loop at its base into which the larger eppendorf could be placed. The wand was then plunged into liquid nitrogen for a few minutes, after which the eppendorf was immediately centrifuged for 5 minutes in a microfuge. The resulting liquid containing the PCR product was collected in the 1ml eppendorf during the spin.

5. A Nen-Sorb column was then used to remove any contaminating proteins, salt and low molecular weight materials from the PCR product. The Nen-Sorb column was set up with an empty syringe attached via an adapter to the top of the column. This was used to push through the various solutions at a flow rate not greater than one drop every two seconds. It was prepared by washing with first 1ml methanol, followed by 1ml Buffer A. Then the PCR product was mixed with 500μl of

Buffer A and applied to the column and eluted with 200μl of 50% iso-propanol. It was then dried with a Speedy Vac, with subsequent resuspention in 20μl of TE buffer.

6. At this point the sample was applied to a 1ml CL6B Sepharose column and centrifuged. The resulting eluent contained the PCR product whilst the dyes from the gel electrophoresis were retained on the Sepharose.
7. A sample of the cleaned PCR product was analysed by 1% (w/v) agarose gel electrophoresis (Section 2.2.2.) and further analysed by Southern blotting (Section 2.2.3.). If a positive hybridization occurred, as determined from the autoradiograph, then the PCR product was prepared for ligation into a suitable vector.

2.2.6 Preparation of PCR Product for Cloning

2.2.6.1 Blunt Ending

This was used to fill in the ends of the PCR product, so that blunt ends were created.

Materials.

- PCR DNA Product from freeze squeeze (section 2.2.5.4).
- Klenow Fragment (carries the 3' to 5' exonuclease activity of the intact DNA polymerase I, but lacks the 5' to 3' activity.)
- Klenow Buffer (Manufacturers supplied with enzyme)
- dNTP's 1.25mM (dATP, dTTP, dCTP, dGTP)

Method

The following were placed in an autoclaved eppendorf 20μl dNTP's, 20μl PCR DNA, 8μl Klenow buffer and 1μl Klenow fragment. The eppendorf was sealed and incubated for one hour at 37°C. The reaction was terminated by the addition of 1μl 100mM EDTA and then 100μl of phenol was added. This was gently mixed and centrifuged for 3 minutes in a microcentrifuge. The aqueous phase was removed and loaded onto a 1ml CL6B Sepharose column, which was then centrifuged in a microfuge for 5 minutes. The resulting eluent contained the PCR product whilst the klenow fragment and any residual phenol were retained by the column. The PCR product was retained for further processing.

2.2.6.2 EcoR1 Digestion Of Klenow Treated PCR Product

After being blunt ended (section 2.2.6.1), the DNA was cut with EcoR1 to prepare it for insertion into the EcoR1 site in the cloning vector.

Materials

- EcoR1(NBL)
- EcoR1 X10 Buffer (NBL)
- PCR Product (from section 2.2.6.1)
- CL6B Sepharose (Pharmacia)

Method

The following were loaded into an eppendorf PCR DNA, 5μl EcoR1 buffer, 2μl or 3μl of EcoR1, sufficient distilled water to bring the reaction volume to 50μl. This was then incubated overnight at 37°C in a waterbath. After which time the products were loaded onto a CL6B Sepharose 1ml column, which was microfuged and the eluent collected. A sample of the reaction was analysed by 1% agarose gel electrophoresis (section 2.2.2).

2.2.7 Preparation of Vectors for Cloning

Initially pre-prepared M13mp19 and pUCmp19 plasmid were used as vectors.

M13mp19 is a single stranded filamentous bacteriophage (7.56kb) and is used because single stranded DNA can be obtained which can be used directly for dideoxy sequencing. Its map is shown in figure 18. pUCmp19 plasmids were developed from M13, by the insertion of M13 into a version of pBR322. Consequently they share a variety of commonly used cloning sites and fragments of DNA can be moved between the two.

pUCmp19 is 2.69kb and lacks the *rop* gene that codes for the small *rop* protein (63 amino acids). This protein enhances the binding between RNA's I and II, which effectively regulates replication and therefore the copy number. Because the protein is absent, pUC plasmids can replicate to very high copy numbers, between 500 and 700. Its map is shown in figure 19.

As the work advanced two types of vector were prepared, a blunt ended vector and a blunt and sticky ended vector.

Materials

- M13mp19 and pUCmp19
- HincII, EcoRI (NBL)
- Restriction Enzyme Buffers (NBL).
- CL6B Sepharose (Pharmacia)
- Tris treated Phenol (Rathburn Chemicals Ltd.)
- 95% (v/v) Ethanol
- Tris-HCl pH 8.3.

Method

1. For the blunt ended vector the following were placed in an eppendorf:

6μg pUCmp19, 4μl of HincII buffer, distilled water to give a final volume of 20μl.

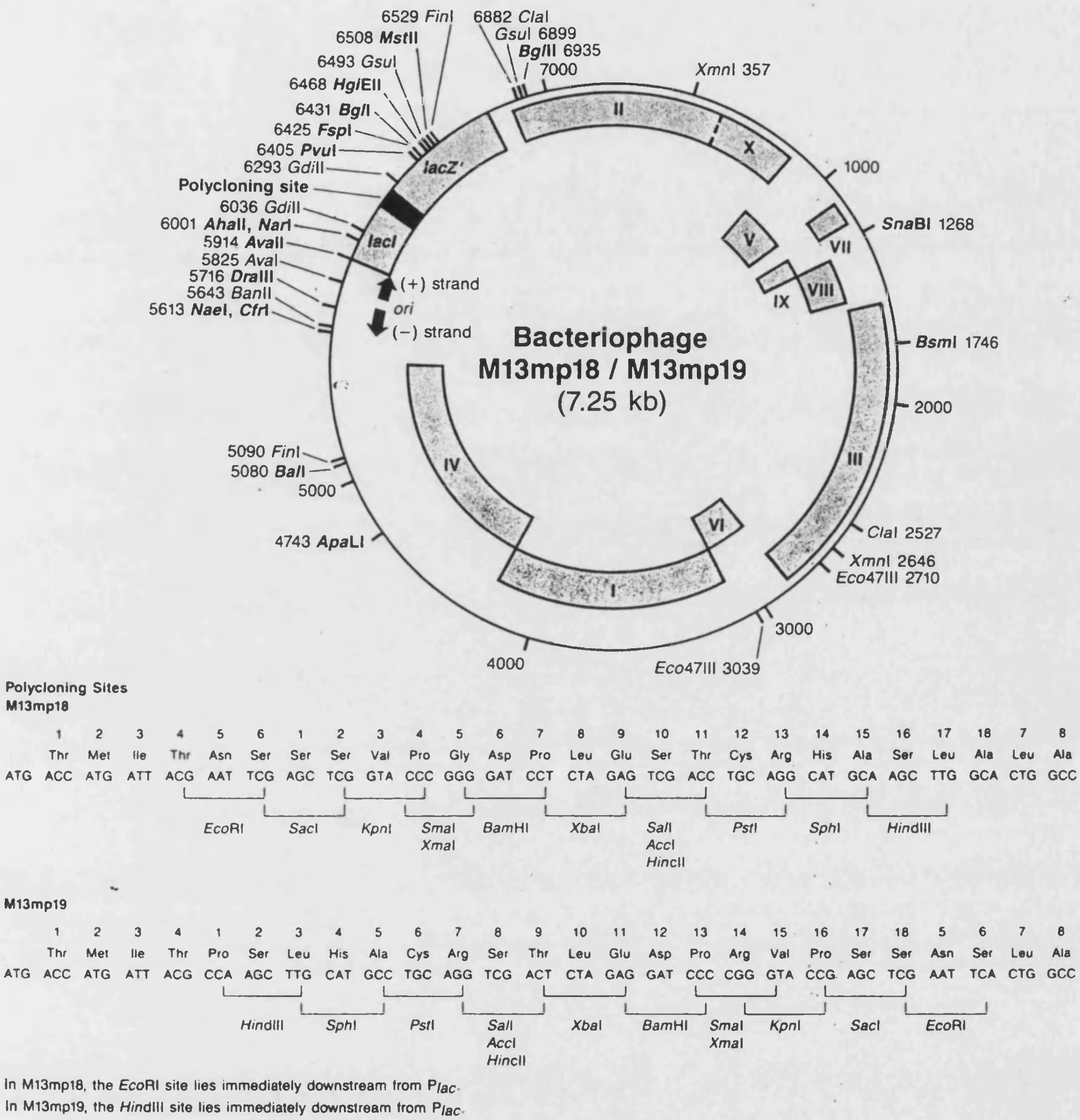
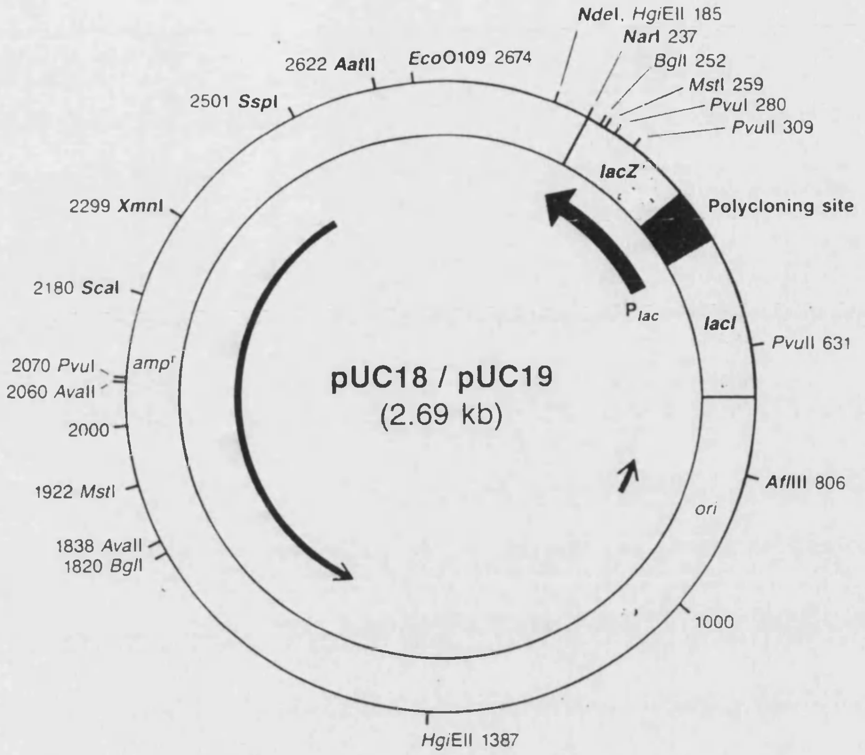


Figure 18. Restriction Map of M13mp19.

(Messing 1983; Norrander et al. 1983; Yanisch-Perron et al. 1985)



Polycloning Sites

pUC18

	1	2	3	4	5	6	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Thr	Met	Ile	Thr	Asn	Ser	Ser	Ser	Val	Pro	Gly	Asp	Pro	Leu	Glu	Ser	Thr	Cys	Arg	His	Ala
ATG	ACC	ATG	ATT	ACG	AAT	TCG	AGC	TCG	GTA	CCC	GGG	GAT	CCT	CTA	GAG	TCG	ACC	TGC	AGG	CAT	GCA

16	17	18	7	8	
Ser	Leu	Ala	Leu	Ala	
AGC	TTG	GCA	CTG	GCC	

EcoRI *SacI* *KpnI* *SmaI XmaI* *BamHI* *XbaI* *SalI AccI HincII* *PstI* *SphI* *HindIII*

pUC19

	1	2	3	4	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	Thr	Met	Ile	Thr	Pro	Ser	Leu	His	Ala	Cys	Arg	Ser	Thr	Leu	Glu	Asp	Pro	Arg	Val	Pro	Ser
ATG	ACC	ATG	ATT	ACG	CCA	AGC	TTG	CAT	GCC	TGC	AGG	TCG	ACT	CTA	GAG	GAT	CCC	CGG	GTA	CCG	AGC

18	5	6	7	8	
Ser	Asn	Ser	Leu	Ala	
TCG	AAT	TCA	CTG	GCC	

HindIII *SphI* *PstI* *SalI AccI HincII* *XbaI* *BamHI* *SmaI XmaI* *KpnI* *SacI* *EcoRI*

In pUC18, the *EcoRI* site lies immediately downstream from P_{lac}

In pUC19, the *HindIII* site lies immediately downstream from P_{lac}

Figure 19. Restriction Map of pUCmp19.

(Messing 1983; Norrander et al. 1983; Yanisch-Perron et al. 1985)

For the blunt and sticky ended vector:

6μg pUCmp19, 2μl HincII and 2μl EcoR1 buffer, distilled water to bring the final volume to 20μl.

Two controls were also set up to check that both the enzymes were active, 0.5μg pUCmp19, 2μl HincII, 2μl HincII buffer and 0.5μg pUCmp19, 2μl EcoR1, 2μl EcoRI buffer. To both, 15μl of double distilled water was added.

2. The tests and controls were incubated at 37°C for two hours and left at 4°C overnight to ensure complete digestion.

3. Once the digests were completed the total sample from each reaction was loaded onto a 0.8% (w/v) agarose gel (section 2.2.2) which was run at 30mA for 6-7 hours. It was then viewed with a UV transilluminator.

4. The bands representing digested pUC, except the controls that were used as markers, were then excised with a scalpel and placed in separate eppendorfs. Each sample of cut pUC was recovered by freeze squeeze (section 2.2.5.4, Number 4.).

5. The resulting liquid was applied to a 1ml CL6B sepharose column, which was microfuged for 5 minutes. After this, the eluent was mixed with 50μl of chloroform and 50μl of Tris-treated phenol before being centrifuged for 2 minutes. The aqueous phase was removed and added to 1ml of 95% (v/v) pre-chilled ethanol. The cut pUC DNA was allowed to precipitate overnight at 4°C. Then the supernatant was aspirated off, and the pellet resuspended in 90μl Tris-HCL pH8.3. The two samples, of blunt ended and blunt and sticky ended vector, were then stored at 4°C until required.

2.2.8 Cloning of Selected DNA

A generalised scheme for the approach to cloning either the PCR DNA or pieces of genomic DNA is shown in Figure 20.

2.2.8.1 Ligation of PCR Product DNA into M13 and pUC

Initially M13mp19 was used as the vehicle for delivering the DNA into Escherichi coli. However, the results were unsatisfactory so pUCmp19 plasmid was used instead.

Materials

- M13 and pUC DNA cut with EcoR1, and pUC previously treated with Sma-1BAP
- PCR DNA (section 2.2.6.2)
- T4 DNA Ligase (NBL)
- x10 Ligase Buffer (NBL):-500mM Tris-HCl pH7.8, 100mM $MgCl_2$, 10mM dithiothreitol, 10mM ATP, 1mg/ml bovine serum albumin.
- 5mM ATP
- CL6B Sepharose (Pharmacia)
- Hexamine cobalt III chloride 10mM stock solution.

Method

Ligation with M13mp19

The following were placed into an autoclaved eppendorf 60μl of PCR DNA, 25μl M13, 5μl x10 Ligase buffer, 5μl 5mM ATP and 5μl T4 DNA Ligase. This was then incubated at 14°C (as recommended by manufacturer) overnight in a waterbath in a cold room. Finally, the mixture was applied to a 1ml CL6B sepharose column, centrifuged in a microfuge and the eluent collected.

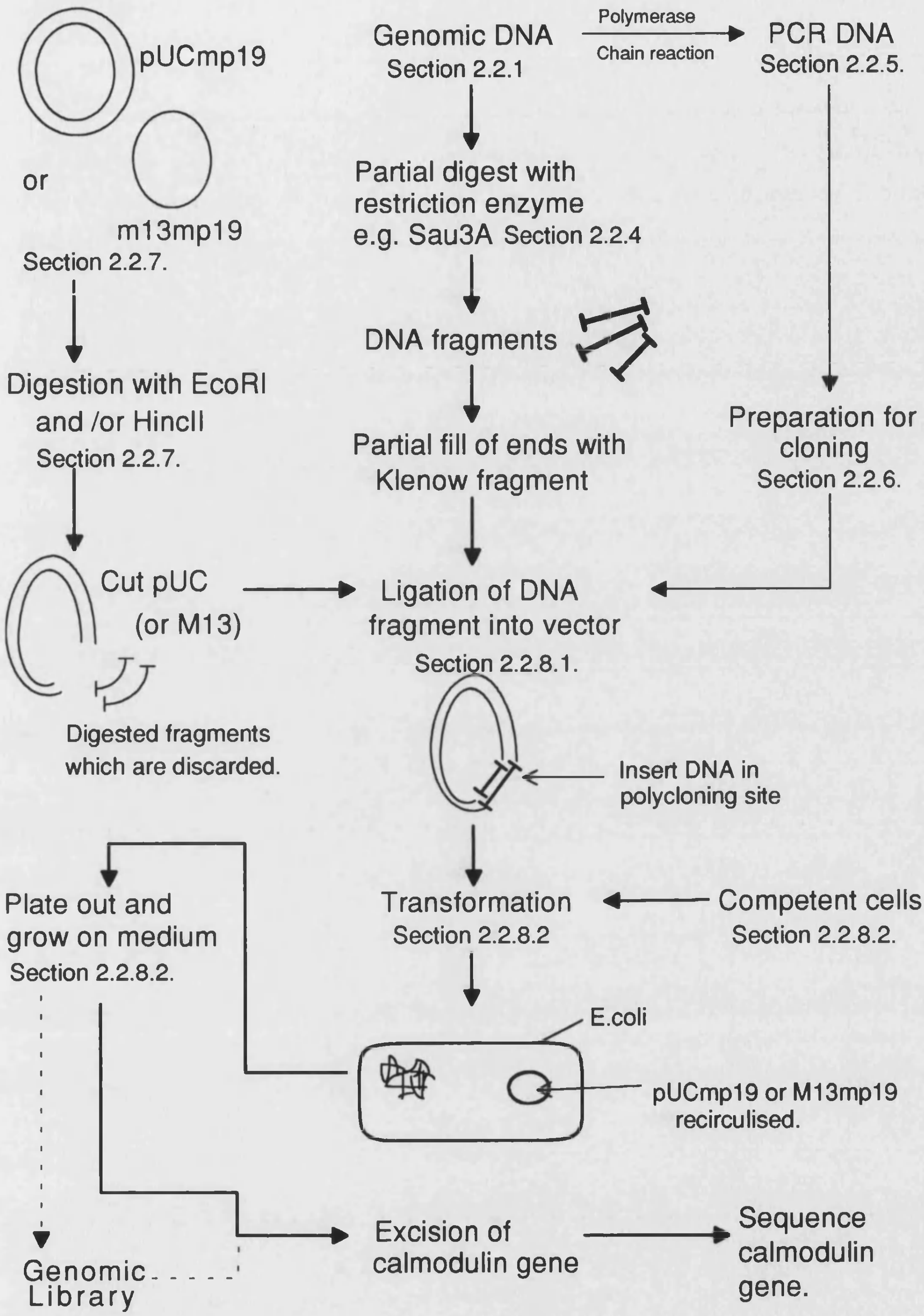


Figure 20. Diagram Showing Scheme for Cloning a Gene

Genomic DNA can be processed with restriction enzymes or amplified by PCR, before ligation into a vector, which is then amplified. A genomic library can be formed and/or a clone carrying the calmodulin gene isolated and sequenced.

Ligation with pUCmp19

The following were placed into an autoclaved eppendorf 1µl to 5µl pUC (equivalent to 10-300ng), 1µl to 30µl PCR DNA, 4µl Ligase buffer, 1µl (1U) T4 DNA ligase, 1µl 5mM ATP and sufficient distilled water to make the final volume to 20µl or 50µl depending on the quantity of PCR DNA. The tubes were incubated at 14°C over the weekend (approximately 63 hours). To increase the efficiency of the ligation reaction, particularly of the blunt ended DNA 1mM hexamine cobalt III chloride was incorporated into the ligation mixture. Hexamine cobalt III chloride increases macromolecular crowding and induces condensation of the DNA into aggregates (Rusche & Howard-Flanders 1985).

2.2.8.2 Preparation of Competent Cells and Transformation

Escherichi coli (strain = TG1*) cells were used as the host bacterium. The PCR DNA that had been ligated into the vector (M13mp19 or pUCmp19)[section 2.2.8.1] was to be infected into the bacterium. M13 transfects E.coli by entering through the bacteria's pili, but the precise mechanism of entry for pUC is unknown. Once incorporated the DNA will be multiplied.

Materials

- Double Yeast Tryptone Broth:- 2% (w/v) tryptone, 1% (w/v) yeast powder and 1.0% (w/v) NaCl.
- Luria Bertini Broth:- 1.0% (w/v) tryptone, 0.5% (w/v) yeast powder and 1.0% (w/v) NaCl at pH 7.5.
- Agar:-1.5%(w/v) Bacto-agar made with either of the above broths.
- Top Agar:-0.7% (w/v) bacto-agar in Luria Bertini broth.
- Lambda Agar:-1.2% (w/v) bacto-agar, 0.25% (w/v) NaCl, 1% (w/v) tryptone autoclaved prior to the addition of 1ml filter sterilized 1M $MgSO_4$ (0.2% w/v).
- Ampicillin

*TG1 Genotype=k12del(lac,pro)supE,thi hsdD5/F'traD36,proAB+,lacIq,lacZ delM15. Gibson (1984) 'Studies on the Epstein-Bar virus genome' PhD Thesis, Cambridge University, England.

- Minimal Media:- K_2HPO_4 1.05% (w/v), KH_2PO_4 0.45% (w/v), $(NH_4)_2SO_4$ 0.1% (w/v) and 0.05% (w/v) tri-sodium citrate. Autoclaved before being brought to 0.02% (w/v) $MgSO_4.7H_2O$, 0.2% (w/v) glucose and 5µg/ml thiamine using filter steralized stock solutions. Finally 1.5% (w/v) solid agar was added.
- 2% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) made in 20mg/ml dimethylformamide.
- 0.1M IPTG (isopropyl-β-D-galactoside) (NBL)
- 100mM $CaCl_2$

Method

Preparation of Competent cells

The method used is based on that described by Maniatis, Fritsch and Sambrook (1989) which is a variation on the method of Cohen, Chang and Hsu (1972).

1. A loop of E.coli was collected from a colony plate and transferred to 5ml Luria Bertini broth. The bacteria were incubated overnight at 37°C on a shaking platform either in a constant temperature room or in an incubator.
2. 500µl was removed from this overnight culture and placed in 50ml of pre-warmed double yeast tryptone broth. They were then incubated at 37°C, on a shaking platform, until the absorbance at 600nm was 0.4. this took approximately 40 minutes.
3. After which time the suspension was rapidly chilled, in a slurry of ice, with constant mixing. When thoroughly cooled, the suspension was transferred to 50ml Falcon tubes and centrifuged at between 2500rpm and 5000rpm at 4°C for 10 minutes. The supernatant was removed and 20ml of chilled 100mM $CaCl_2$ used to resuspend the pelleted bacteria. They were then left on ice for 20 minutes before being centrifuged at 5000rpm for 10 minutes at 4°C. The supernatant was

carefully decanted and the pelleted bacteria resuspended in between 1ml and 3 ml of chilled 100mM $CaCl_2$. They were then chilled on ice for 30 minutes before being aliquoted out into eppendorfs in 300µl portions.

Transformation

Cells were transformed with uncut pUC or M13 DNA, cut and re-ligated vector DNA, and vector containing insert PCR DNA.

4. The vector DNA (between 1 to 10ng is the ideal quantity) was added to 300µl of the aliquoted competent cells, which were allowed to stand for up to one hour on ice. Then they were heat shocked for 2 to 3 minutes at 42°C and again left on ice for 30 minutes. After this time, to each 300µl of cells 300µl of Luria Bertini broth was added. They were then incubated at 37°C for 30 minutes for M13 and 60 minutes for pUC. The transformed cells were then ready to be plated out.

For the M13 transformed cells:

Lambda agar plates were prepared. Approximately 2ml of top agar was poured into Luckhams tubes which were then placed in a warming block set at 37°C or 42°C. To each 300µl of the transformed cells 50µl X-gal, 10µl IPTG and 10µl of extra non-transformed cells (from the culture in Double yeast tryptone) were added. This mixture was then added to the warmed top agar, which was quickly mixed and poured onto the lambda plates. The plates were allowed to set and air-dry before being placed in an incubator.

For pUC transformed cells:-

Luria Bertini agar plates were prepared containing 0.04% (w/v) ampicillin. To the transformed cells 50µl X-gal and 10µl IPTG were added and then they were poured onto the centre of the plates and spread as evenly as possible across the surface. These were allowed to air dry before being incubated.

Plates were then incubated at 37°C for 24 hours. If the E.coli were transformed with either pUC or M13, then blue/green colonies would form. This is due to α-complementation of the β-galactosidase gene product of both the E.coli and the vector. E.coli contain the genetic code for the carboxy terminal of β-galactosidase whereas pUC and M13 possess the sequence for the amino terminal fragment. The presence of pUC or M13 in the E.coli means that the two can complement each other so giving rise to an active protein, which enables the bacteria to metabolise the galactoside substrates present in the agar. If a piece of insert DNA is present, in the vector, then the β-galactosidase gene is disrupted. Consequently the bacteria cannot utilise galactoside substrates resulting in white or pale blue colonies (Ullman 1967; Horwitz 1964).

2.2.8.3 Small Scale Plasmid Preparation

Once a white colony (containing PCR DNA) was identified, the cells were amplified to produce more plasmid DNA. The same method was also used to produce plasmid DNA for use as a vector and is a variation of that detailed by Maniatis, Fritsch and Sambrook (1989) which is adapted from Godson and Vapnek (1973).

Materials

- Luria Bertini Broth containing 100μg/ml ampicillin.
- 20% (v/v) Glycerol
- Glucose/Tris Buffer:- 50mM glucose, 25mM Tris-HCl pH8.0.
- 3m Sodium Acetate pH 4.8
- Lysozyme Buffer: 50mM glucose, 25mM Tris-HCl pH 8.0, 10mM EDTA, 2mg/ml lysozyme.

- 0.2N NaOH, 1% (w/v) Sodium dodecyl sulphate
- Ice Cold 95% and 70% (v/v) Ethanol

Method

1. The white plaques (from section 2.2.8.2) were picked out and placed in 5ml of Luria Bertini broth containing ampicillin and incubated at 37°C overnight on a shaking platform either in a constant temperature room or in an incubator.
2. After this time 100µl of the cultured cells were removed and diluted with 20% (v/v) glycerol and frozen at -20°C.
3. The remaining cells were centrifuged at 5000rpm for 5 minutes and the supernatant removed. The pelleted cells were resuspended in 20µl glucose /Tris buffer followed by sufficient lysozyme buffer to give a final volume of 100µl. The cells were then mixed for 10 minutes on ice before the addition of 200µl 0.2N NaOH, 1% (w/v) sodium dodecyl sulphate. They were agitated and left to stand for 10 minutes on ice. After this 150µl of 3M sodium acetate was added to the cells, which were placed at -20°C for 10 minutes to allow a white precipitate to form. Following which the cells were centrifuged for 15mins at 15,000rpm, at 4°C.
4. The resulting supernatant was removed and mixed with 95% (v/v) ice cold ethanol and allowed to stand on ice for 10 minutes. The precipitated DNA was then pelleted by centrifugation in a microfuge, for 5 minutes, and the supernatant discarded. To the pellet 500µl 70%(v/v) chilled ethanol was added and the mixture dried in a Speed-Vac.

5. The resulting pellet was resuspended in double glass distilled water and a sample taken for analysis by agarose gel electrophoresis (section 2.2.2). A sample of the DNA was then treated with restriction enzymes, as previously described (section 2.2.4).

2.2.9 RNA Isolation Procedures

As an alternative approach to cloning the calmodulin gene from genomic DNA it was decided to isolate the RNA, from which the mRNA could be isolated. A cDNA library could then be produced which could be probed for the calmodulin gene, the gene extracted and sequenced. A diagram showing the general scheme is shown in figure 21. Also the mRNA could be inserted into a translation vector, e.g. Xenopus laeviis oocytes, and the calmodulin **protein** isolated and sequenced.

The method chosen to isolate RNA was based on the single step acid guanidinium thiocyanate-phenol-chloroform extraction of Chomczynski and Sacchi 1987.

Materials

- Diethyl Pyrocarbonate (DEPC) Treated Water (0.1% (w/v) DEPC in autoclaved double distilled water is stirred for 15 minutes, followed by incubation at 70°C for one hour. This degrades the DEPC to ethanol and CO_2)
- Solution D:- 4M guanidinium thiocyanate, 25mM sodium citrate pH7, 0.5% (w/v) sarcosyl,
- 0.1M 2-Mercaptoethanol (in DEPC treated water)
- 2M Sodium Acetate pH4.0
- 0.5% Sodium dodecyl sulphate (in DEPC water)
- Phenol (Rathburn Chemicals Ltd.)
- Chloroform:Iso-Amyl Alcohol (49:1)
- Iso-Amyl Alcohol
- 75% (v/v) Ethanol

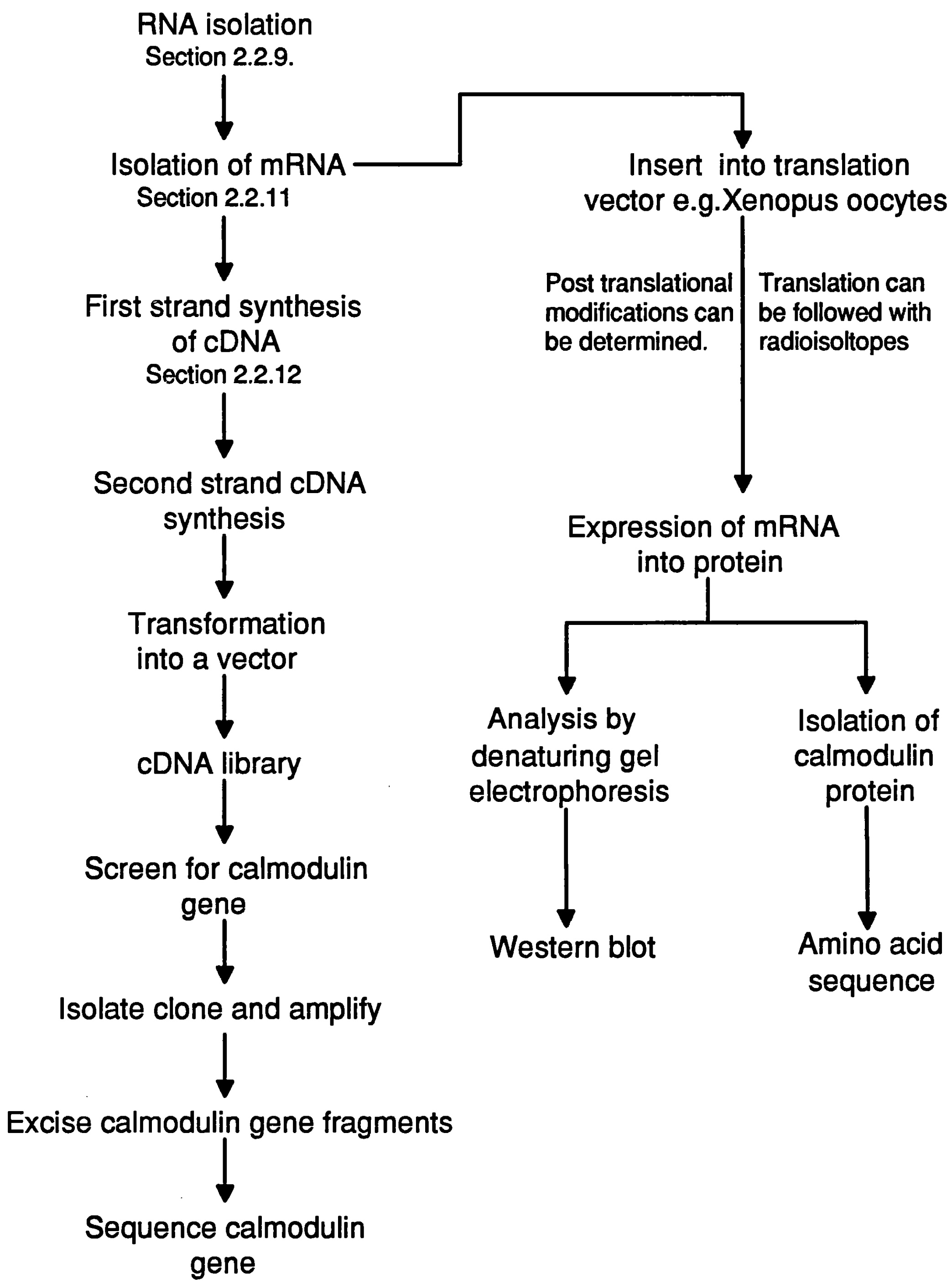


Figure 21. Scheme For cDNA Cloning And Sequencing

Messenger RNA isolated from RNA can be used to generate complementary DNA (cDNA) which is then inserted into a vector, amplified and a cDNA library produced. mRNA can also be translated and its products studied.

Method

1. 3g of fresh tapeworms (isolated according to section 2.1) were homogenised in 30ml of solution D. Then the following were added sequentially with mixing 3ml 2M sodium acetate, 30ml saturated phenol and 6ml chloroform-isoamyl-alcohol (49:1). This was left to stand for 15 minutes on ice. After this time, the mixture was centrifuged at 10,000g for 20 minutes, at 4°C. The aqueous phase was removed and transferred to a clean tube and 30ml of iso-propanol was added. The tubes were placed at -20°C overnight and then centrifuged for 20 minutes at 10,000. The supernatant was discarded and the pellet resuspended in 300µl of solution D and transferred to an autoclaved eppendorf. From which the RNA was precipitated at -20°C by the addition of 1 volume of iso-propanol, for one hour.
2. Following this the RNA mixture was centrifuged in a microfuge for 10 minutes, at 4°C, and the supernatant removed whilst the pellet was resuspended in 75% (v/v) ethanol and centrifuged for 10 minutes at 10,000g and 4°C. The ethanol was then carefully aspirated off and the pellet resuspended in 6mls of 0.5% (w/v) sodium dodecyl sulphate.
3. This was then heated at 65°C for 10 minutes to dissolve the RNA before it was stored at -70°C until required.
4. 10µl of the RNA solution from above was mixed with 990µl of DEPC treated water in a 1cm^2 1ml quartz cuvette. The mixture was then scanned between 200nm and 300nm and the absorbance recorded. From which the RNA concentration was calculated where one absorbance unit at 260nm=40µg/ml RNA or 37µg/ml single stranded RNA.

2.2.9.1 Removal of Sodium Dodecyl Sulphate from RNA Preparations

The sodium dodecyl sulphate was removed from RNA samples by the following method.

Materials

- 5M NaCl
- 95% and 70% (v/v) Ethanol
- TE Buffer: 10mM Tris-HCl pH8.0, 1mM EDTA pH8.0.

Method

The RNA sample (from section 2.2.9.) was brought to 0.2M NaCl by the addition of 5M NaCl. To this was added two volumes of 95%(v/v) ethanol followed by overnight storage at -20°C to allow the RNA to precipitate out of solution. This was then centrifuged in a bench centrifuge for 10 minutes at 5000rpm and the supernatant removed by aspiration The pellet of RNA was resuspended in one to two volumes of 70% (v/v) ethanol and the mixture stored at -20°C for 1 to 2 hours. Following which it was centrifuged for 15 minutes at 5000rpm and the supernatant removed by aspiration. The pellet was dissolved in TE buffer pH8.0

2.2.10 Gel Electrophoresis of RNA samples

Isolated samples of RNA were analysed by both, agarose and polyacrylamide gel electrophoresis.

2.2.10.1 Agarose Gel Electrophoresis of RNA samples

The method used was based on that by Lehrach et al. 1977; Goldberg 1980; Maniatis, Fritsch & Sambrook 1989.

Materials

- x10 Running Buffer:- 0.2M 3'N-morpholiopropane-sulphonic acid (MOPS) pH7.0, 50mM sodium acetate, 5mM EDTA pH8.0 (in DEPC treated water)
- Agarose (electrophoresis grade)
- Sample Dye:- 30% (w/v) Ficoll, 1mM EDTA, 0.25% (w/v) Bromophenol blue and 0.25% (w/v) Xylene cyanole.
- Deionized Formamide:- 100ml formamide mixed with 5g mixed bed resin (BioRad AG501-X8) and stirred for 1 hour. Then filtered through Whatman No.1 filter paper and stored at -20°C.
- Deionized Formaldehyde (prepared as for formamide)
- Ethidium Bromide 10mg/ml (made with DEPC treated water
- RNA Size Markers (Boehringer Mannheim)
- RNA samples from section 2.2.9 or 2.2.9.1.

Method

1. To 1g agarose, 10ml of x10 Running buffer was added together with 85ml of DEPC treated water. This was then microwaved in a domestic oven until the agarose had dissolved it was then cooled to 55°C. At this point 5.4ml of deionized formaldehyde and 10µl of ethidium bromide were added and the mixture quickly poured into a gel cast where it was left to set.
2. Whilst the gel was setting the samples were prepared as follows 10µg of RNA was mixed with 2µl of x10 Running buffer, 10µl freshly deionized formamide, 3.5µl of deionised formaldehyde and 2µl of sample dye. They were then heated in a waterbath at 55°C for 15 minutes.
3. By this time the gel had set, so it was transferred to a gel tank that had been filled with x1 running buffer. The samples were then loaded and the gel run for approximately 1 hour at 100mA so that the RNA entered the gel. At this point

more running buffer was added so that the gel was fully immersed and electrophoresis resumed at 8mA. It was run until the dyes were approximately 1cm from the end of the gel. It was viewed with a UV transilluminator and photographed with a Polaroid camera.

2.2.10.2 Polyacrylamide Gel Electrophoresis of RNA Samples

Materials

- Solid Acrylamide
- Solid Bisacrylamide
- Deionized Formamide (see materials section 2.2.9.2)
- Solid $Na_2HPO_4.7H_2O$
- Solid $NaH_2PO_4.H_2O$
- Solid Ammonium Persulfate
- (N,N,N,N-Tetramethylethylene-diamine) [TEMED]
- Whatman No.1. Filter Paper
- Running Buffer:- 16mM Na_2HPO_4, 4mM NaH_2PO_4 pH7.5
- Staining Solution:- 0.02% (w/v) methylene blue, 10mM Tris-acetate pH8.3.
- RNA sample from section 2.2.9 or 2.2.9.1.
- Marker Dye:- 30% (w/v) ficoll, 1mM EDTA, 0.25% (w/v) bromophenol blue and 0.25% (w/v) xylene cyanole.

Method

1. A 4% gel was prepared as follows: 2.55g acrylamide and 0.45g bisacrylamide were mixed with 74ml deionized formamide. To this was added 1ml of DEPC treated water that contained 0.32g $Na_2HPO_4.7H_2O$, 0.04g $NaH_2PO_4.H_2O$ and 0.1g ammonium persulfate. After mixing the solution, it was filtered through Whatman No1. filter paper. Then 50µl of TEMED was added with mixing before being poured into a gel cast. A comb was inserted and the gel was allowed to polymerise. Once the gel had set the comb was removed and the wells rinsed

with DEPC treated water. The gel was then placed into the gel tank, which was filled with x1 running buffer.

2. The sample containing RNA (10µg) was mixed with 2.5µl of deionised formaldehyde and 5µl of marker dye. Following which it was heated at 70°C for 10 minutes before being loaded into the gel.
3. The gel was run at 150V for one and a half hours after which time it was removed and placed in staining solution overnight. Finally it was destained with DEPC treated water and placed on Whatman 3MM paper and allowed to dry at room temperature.

2.2.11 Isolation of mRNA from Total RNA

Materials

- Oligo (dT) Cellulose Type 7 (Pharmacia 275543)
- Siliconized Glass Wool (donated)
- 0.1M NaOH
- Application Buffer:-10mM Tris-HCl pH7.5, 1mM EDTA, 0.5M NaCl
- Wash Buffer:- 10mM Tris-HCl pH7.5, 1mM EDTA, 0.1M NaCl
- 0.5% (w/v) Sodium dodecyl sulphate
- TE Buffer:- 10mM Tris-HCl pH7.4, 1mM EDTA
- TE Buffer solutions containing 5M, 2M and 1M NaCl
- 5mM EDTA
- 95% and 50% (v/v) Ethanol
- Ethidium Bromide 1µg/ml
- All solutions made with DEPC treated water

Method

1. 0.5g oligo (dT) cellulose was mixed in 2ml of DEPC double glass distilled water and poured into a 0.6cm autoclaved mini-column, which was plugged with

siliconised glass wool. Two such columns were prepared and then washed with 5 bed volumes of 0.1M NaOH followed by washing with application buffer until the eluent was pH7.5.

2. The total RNA (pelleted)[section 2.2.9.1] was suspended in 0.5ml of TE buffer and heated at 65°C for 5 minutes and then cooled on ice. 0.5ml of 1M NaCl was added and mixed, before the RNA sample was applied to one of the columns. The column was run under gravity and the eluent collected, heated at 65°C for 5 minutes and then reapplied to the same column. This was subsequently washed with 5-10 column volumes of application buffer, followed by 5 volumes of wash buffer. This treatment effectively removed RNA that did not contain polyA tails. Bound mRNA (polyA+) was eluted with 3 volumes of TE buffer and collected in 200μl fractions.

   Those fractions that contained RNA were identified by placing 1 to 3μl of each fraction onto a piece of clingfilm and to each spot 20μl of ethidium bromide solution was added. The clingfilm was then viewed under with a UV transilluminator. Fractions containing RNA fluoresce and so those fractions that contained RNA were pooled, heated at 65°C for 5 minutes and then chilled rapidly on ice.

3. To this, one volume of 1M NaCl was added and mixed and the resulting solution applied to the second oligo (dT) column. The eluent was collected and reapplied to the column, in the same way as for the first column. Washing was carried out first with 5-10 column volumes of application buffer and then with 5 column volumes of wash buffer. Again this treatment removes any RNA that lacks a polyA tail. Finally the mRNA (containing polyA tail) was eluted with TE buffer

and collected in 200μl fractions. These were then checked for the presence of RNA, by UV fluorescence as before and those fractions that contained RNA pooled. To this was added, 0.2 volumes of 2M NaCl and 3 volumes of cold 95% (v/v) ethanol after which they were chilled at -20°C for a minimum of 2 hours.

4. This mixture was then microfuged for 10 mins at 4°C, the supernatant removed and the pellet briefly dried by aspiration. It was then redissolved in 20μl of TE buffer.

The concentration of RNA was determined by scanning the absorbance from 300nm to 200nm (as described in section 2.2.9.). The mRNA was analysed by agarose gel electrophoresis (section 2.2.10.1.).

2.2.12 First Strand cDNA Synthesis

Materials

- 10x Buffer:- 500mM Tris-HCl pH8.3, 400mM KCl, 60mM $MgCl_2$ and 10mM Dithiothreitol
- 0.1M Dithiothreitol
- 10mM dNTP's (dATP, dCTP, dGTP, dTTP)
- RNAGUARD™(Pharmacia) [RNAase inhibitor from human placenta]
- Oligo-(dT_{12-18}) prepared as a 1μg/μl mixture in DEPC treated water. (Pharmacia)
- Maloney-Murine Leukaemia Virus Reverse Transcriptase (Gibco BRL 200U/μl)
- AT-γ-^{32}P in ethanol:water 1:1 (Amersham. 2mCi/ml; >5000 Ci/mmol)

Method

1. The following were mixed in an autoclaved eppendorf: 2μl x10 buffer, 2μl 0.1M dithiothreitol, 1μl 10mM dNTP's, 0.5μl oligo(dT_{12-18}), 0.5μl RNAguard™, 1μl

Reverse transcriptase, and 12.5μl of distilled water. To this was added, 0.5μl of 2mg/ml mRNA from section 2.2.10.

2. A second eppendorf tube was prepared into which 0.5μl (equivalent to 50μCi) of AT-γ-^{32}P was placed, and dried in a Speed-Vac.

3. 5μl was removed from the first eppendorf tube and placed into the second eppendorf. This will be used to check that the cDNA is correctly synthesised and that the mRNA has not degraded.

4. Both tubes were then incubated at 37°C for 1 hour in a waterbath and to each tube 80μl of TE buffer was added before they were stored at 4°C until required.

5. The contents of the second tube, containing AT-γ-^{32}P, was put through a sephadex G25 spun column, as described in section 2.2.3.1., to remove any unincorporated AT-γ-^{32}P. It was then retained for alkaline agarose gel electrophoresis (section 2.2.12.1)

2.2.12.1 Alkaline Agarose Gel Electrophoresis of cDNA.

This was used to check the synthesis of cDNA using the labelled sample from section 2.2.12. It can also be used to identify the presence of 'hairpins' which are secondary structures which can form when the newly polymerised first or second strand of DNA 'snaps back' onto itself to form an anti-parallel double helix.

Materials

- x10 Alkaline Buffer:- 0.3M NaOH, 20mM EDTA in DEPC treated water
- T4 Polynucleotide Kinase (NBL)
- Kinase Buffer (NBL)
- Agarose
- HindIII DNA
- 25mM NaOH
- Whatmans 3MM Filter Paper
- X-Ray Film (Fuji RX-100)

- AT-γ-^{32}P in ethanol:water 1:1(Amersham 2mCi/ml,>5000Ci/mol)
- 2x Loading Buffer:- 20% (v/v) glycerol, 4.6%(v/v) bromophenol blue (using a saturated solution)

Method

1. First the Lambda HindIII DNA was radiolabelled with AT-γ-^{32}P and so the following reaction was set up in an eppendorf: 0.5μl lambda HindIII DNA (112 to 115ng), 0.5μl (equivalent to 5μCi), 5μl x10 kinase buffer, 3μl polynucleotide kinase (18U) and double glass distilled water to give a total reaction volume of 30μl. This reaction mixture was incubated at 37°C for 1 hour and then put through a G25 spun column to remove any unincorporated AT-γ-^{32}P as previously described (section 2.2.3.1). It was then stored at 4°C until required.
2. Simultaneously, the agarose gel was cast on a glass slide measuring 5cm x 7.5cm, which had been washed with DEPC treated water followed by ethanol. First a comb was placed above the glass slide, at one end, and held in place by two bulldog clips on either side. A gap of 1mm was left beneath the base of the comb and the surface of the slide. Then 0.8g of agarose was dissolved in 72ml of DEPC treated water in a domestic microwave and allowed to cool to 55°C. At which point 8ml of x10 alkaline buffer was added. Using 10ml of this solution at a time, the gel was carefully poured in layers onto the slide. Once set the gel was placed in the gel tank.
3. To the HindIII DNA (from 1) and the radiolabelled cDNA sample (section 2.2.12) an equal volume of x2 loading buffer was added before they were loaded into the gel. This was initially run at 100mA, until the samples had entered the gel and

then the gel was run at 5mA overnight. After which the gel was removed from the tank and blotted dry with Whatmans 3MM filter paper, wrapped in clingfilm and placed in a film cassette, with a piece of x-ray film placed over the gel. The cassette was then stored at -70°C for two days and the autoradiograph developed.

2.3 Protein Biochemistry

2.3.1 Development of an Isolation Procedure for Calmodulin

Calmodulin, as already stated in the introduction, is a highly conserved protein. The majority of cellular calmodulin is soluble, relatively heat stable and has an isoelectric point of 3.9. (Dedman & Kaetzel 1983). It is present in varying quantities in different cell types, consequently for each tissue a unique isolation procedure has to be developed. In general, large quantities of tissue are required to obtain substantial quantities of calmodulin that can be used for further investigation. However, due to the difficulty of producing vast quantities of tapeworms a small scale isolation procedure had to be developed using the minimum number of steps. Because of this it was decided to develop the best isolation procedure using pig thymus, a tissue that has many physical similarities to tapeworm tissue but which is more readily available.

Materials:-

The materials listed are those used in all the isolation procedures tried.

- Pig thymus (from local slaughter house)
- Homogenisation Buffer:- 10mM Imidazole pH6.1; 5mM EGTA or 5mM EDTA. Sometimes 1mM 2-Mercaptoethanol and 0.5mM PMSF was added.
- 4% (w/v)Trichloroacetic Acid
- Solid Ammonium Sulphate
- 0.5N H_2SO_4 containing 50% (w/v) ammonium sulphate
- 1M and 50mM Tris-HCl
- Concentrated Hydrochloric Acid
- Solid DEAE52 cellulose swollen in double distilled water (Whatmans)
- Dialysis/Visking Tubing (Medicell)

- DEAE52 Buffers:-
  a) 10mM Imidazole, 0.1mM EDTA pH 6.1
  b) 10mM Imidazole pH6.1
  c) 10mM Imidazole, 0.15M NaCl, pH6.1
  d) 10mM Imidazole, 0.4M NaCl pH6.1
  e) Regeneration solutions: 0.5N NaOH and 0.5N HCl.
  Sometimes 0.02% (w/v) sodium azide was included in the buffers as a bacteriostat.
- Phenyl Sepharose pre-swollen supplied in 20% (v/v) Ethanol (Pharmacia)
- Phenyl Sepharose Buffers:-
  a) 50mM Tris pH7.5, 0.1mM $CaCl_2$
  b) 50mM Tris pH7.5, 0.1mM $CaCl_2$, 0.5M NaCl
  c) 50mM Tris pH7.5, 1mM EDTA, 1mM EGTA
  d) Regeneration solution: 6M UREA
- Amicon Concentrator:- 2ml, molecular weight cut off 10kD(Amicon.)

Methods

The methods developed utilise the calcium dependent hydrophobic properties of calmodulin (section 1.1.1) by using a hydrophobic gel matrix, phenyl sepharose as the key isolation step (Gopolakrishna & Anderson 1982). The principle of calmodulin binding to phenyl sepharose is that calcium/calmoduin complexes are applied to the column. In this state, calmodulin exposes hydrophobic residues that can interact with the phenyl Sepharose. A series of high salt washes ensures the removal of contaminating proteins whilst strengthening the calmodulin:phenyl Sepharose interaction. Finally, calmodulin is removed by the addition of a calcium chelator, which induces a conformational change in calmodulin so freeing it from the gel matrix.

Four major protocols were tried and are described here. However, the more subtle changes and variations tried whilst developing the isolation procedure will be discussed in the result's chapter.

2.3.1.1 Method One Heat Treatment and Phenyl Sepharose Chromatography

Initially a two step method was tried based on a protocol by Dedman and Kaetzel. (1983) and Gopolakrishna and Anderson (1982).

1. Tissue (pig thymus or frozen tapeworms) to be used for calmodulin isolation was cut into 1cm^3 pieces. These were placed immediately into an approximately equal volume of homogenising buffer, placed into an omnimix homogeniser and homogenised on ice in 5x1 minute bursts with intervals of 5 minutes. An imidazole buffer was chosen over citrate or acetate buffers because of its low pKa of 6.9. The presence of EGTA or EDTA is essential to ensure that all the calcium ions are chelated leaving calmodulin in a calcium free state. Consequently, it remains in a soluble form and is largely unable to bind to other proteins. No protease inhibitors were used here as a heat step was to be included which will inactivate most proteases.

   The homogenate was then centrifuged at 20,000g for 20 minutes at 4°C after which the supernatant was carefully removed and filtered through four layers of muslin cloth to remove any lipid. The pellet was resuspended in homogenising buffer, blended in the homogeniser for 5x1 minutes, on ice, and centrifuged at 20,000g for 20 minutes at 4°C. Both supernatants were combined and the second pellet retained for later analysis.

2. The supernatant was then rapidly heated using a domestic microwave to 90^{0}C followed by rapid chilling to 4^{0}C using an ethanol dry ice mixture. This step will

denature many proteins including the proteases, and makes use of the fact that calmodulin is heat stable (Dedman & Kaetzel 1983). The mixture was then centrifuged at 20,000g for 20 minutes, at 4^0C, and the supernatant retained. Sometimes the supernatant was cloudy and was re-centrifuged at 20,000g for 20 minutes, at 4^0C. This second supernatant was then brought to pH7.5 with 50mM Tris, and to 6mM $CaCl_2$ using either solid $CaCl_2$ or a stock 1M solution.

3. Phenyl Sepharose Chromatography -

The clear solution from above was applied to a Phenyl-Sepharose column (10cm in height, by 1.5cm in diameter) and allowed to run through under gravity. The column was first washed with buffer A, to remove non-binding proteins, until the absorbance (1cm) at 280nm was less than 0.05. Then it was washed with buffer B (high salt) until the absorbance at 280nm was again less than 0.05. Finally the calmodulin was washed from the column with buffer C (containing EDTA/EGTA) until most of the protein was eluted and the absorbance had returned to 0.05 at 280nm. The column was regenerated by application of 5 to 10 column volumes of solution D.

Throughout the washing by buffers A, B, C and solution D 4ml fractions were collected and the absorbance measured at 280nm and 260nm. Proteins generally have an absorption maximum at around 280nm due to tyrosine and tryptophan residues; whereas nucleic acids have an absorption maxima of ≈255-260nm due to purines and pyramidine bases. Consequently measuring both gives an indication of the purity of the solution (Robyt & White 1987). Initially due to calmodulins low phenylalanine:tyrosine ratio the absorbance was also measured at 253nm, which is the isobestic point of calmodulin's UV absorbance spectrum.

This gives a more reliable indication of the quantity of calmodulin, in a solution, because the absorbance of calmodulin is dependent on the ionic strength of the solution and the degree of calcium binding (Klevit 1983). See Table 3, section 1.1. However, it was found after several repetitions of this procedure that the A280 was sufficient indication of calmodulin's presence.

Fractions that formed a peak in the elution profile were pooled and concentrated by resting dialysis sacs on solid polyethylene glycol 20,000. Once concentrated to 1 or 2ml the samples were concentrated with Amicon concentrators in a centrifuge according to manufacturers' instructions.

The protein content was determined by Coomassie blue assay as described in section 2.3.2., whilst the proteins were analysed by denaturing gel electrophoresis (section 2.3.3.).

4. DEAE52 Ion-Exchange Chromatography

A 2.5x 10cm column of DEAE52 cellulose was pre-equilibriated with buffer-A before the phenyl sepharose fractions thought to contain calmodulin were loaded. The column was then washed with buffer-B until the absorbance, at 280nm, was less than 0.05. A second wash was performed using buffer C until the absorbance was again less than 0.05. Finally the calmodulin was eluted using buffer D. Fractions were collected throughout and pooled before being analysed for protein content by Coomassie blue (section 2.3.2.) and by polyacrylamide gel electrophoresis (section 2.3.3). The column was regenerated by alternate batchwise washing with 0.5N HCl and 0.5N NaOH as per manufacturers' instructions.

2.3.1.2 Method Two Ammonium Sulphate and Phenyl Sepharose Chromatography

A method was tried using ammonium sulphate precipitation instead of the heat treatment step used in method 1 (section 2.3.1.1). Ammonium sulphate has the advantage of concentrating the sample and removing large quantities of contaminating proteins. It is also a better method if there is a high level of phenolics and/or neutral proteases in the sample (Anderson 1983).

First the tissue was homogenised and centrifuged as in method 1 (Section 2.3.1.1.). The resulting supernatant was brought to 50% saturation, by the addition of solid ammonium sulphate (using tables from Robyt & White 1987) and stirred for one hour on ice. The pH was maintained at pH7.0 with 1M Tris-HCl and centrifuged at 13,000g for 30 minutes. The supernatant was retained and the pellet discarded. Following this an isoelectric precipitation was performed on this supernatant by adjusting the pH to 4.0 with a solution of 55% ammonium sulphate in 0.5N sulphuric acid. This was then stirred for 1-3 hours at 4^0C. After which the mixture was centrifuged for 1 hour at 13,000g at 4^0C, the pellet retained and resuspended in homogenising buffer whilst the pH was adjusted to 8.0 using 4M NaOH. This solution was then centrifuged at 20,000g for 20 minutes and the resulting supernatant subjected to phenyl Sepharose chromatography, and if necessary further purification with DEAE52 chromatography as described in method 1 (section 2.3.2.1). The protein content was determined by Coomassie blue assay and the samples analysed by gel electrophoresis (section 2.3.2 and 2.3.3).

2.3.1.3 Method Three TCA, $NH_4(SO_4)_2$ and Phenyl Sepharose

It was suggested by H.Vogel (1991) that using a trichloroacetic acid (TCA) precipitation step may yield greater quantities of calmodulin than the first two methods tried.

The tissue (pig thymus or tapeworm) was homogenised and centrifuged as in method one. (section 2.3.1.1.) Both the final supernatant and pellet were each suspended in 3 volumes of 4% trichloroacetic acid and mixed before centrifugation at 22,000g, at 4°C. The resulting supernatants from the two samples were discarded and the two pellets solubilized in homogenising buffer and combined. The pH was adjusted to pH 7.5 with 4M NaOH prior to being centrifuged at 22,000g. The supernatant was retained and subjected to ammonium sulphate precipitation, followed by an isoelectric precipitation as detailed in method 2 (section 2.3.1.2.). Following this, the resulting pellet was resuspended in homogenising buffer before being subjected to DEAE52 chromatography, followed by phenyl sepharose chromatography as previously described (section 2.3.1.1.). Samples and fractions obtained were analysed for protein content and quantity (sections' 2.3.2 and 2.3.3.).

2.3.1.4 Method Four Final Method

Finally a method was developed which took into account the problems and the time required from the previous three methods.

1. Homogenisation of tissue (pig thymus or tapeworm) in homogenising buffer on ice in 5x1 minute bursts with 5 minute intervals. Followed by centrifugation at 20,000g for 20 minutes, at 4°C. The pellet was resuspended in homogenising

buffer, blended and re-centrifuged at 20,000g for 20 minutes. The resulting supernatants were combined and filtered through four layers of muslin cloth, before ammonium sulphate precipitation (55%) at pH7.0. This mixture was centrifugated at 10,000g, the pellet discarded and the supernatant retained. This was followed by isoelectric precipitation of the supernatant by adjusting the pH to 4.0 with a solution of 55% (w/v) ammonium sulphate in 0.5N sulphuric acid. This was stirred for 1 to 3 hours before centrifugation at 13,000g for one hour. The supernatant was discarded whilst the pellet was resuspended in homogenisation buffer and brought to pH8 with 4M NaOH. The mixture was then re-centrifuged at 13,000g for 20 minutes.

2. DEAE52 Chromatography was performed on the supernatant (as previously detailed in section 2.3.1.1.). The final wash containing the calmodulin was dialysed against several changes of 50mM Tris pH7.5, at $4^{0}C$.
3. Following this the dialysate was subjected to Phenyl Sepharose chromatography (as described in method 1, section 2.3.1.1.).

Fractions were collected throughout and stored for further analysis.

2.3.1.5 G50 Size Exclusion Chromatography

In an attempt to obtain a homogeneous calmodulin fraction, G-50 Sephadex size exclusion chromatography was used on the final samples of calmodulin obtained from the isolation methods in 2.3.1.1. to 2.3.2.4.

Materials

- Solid Sephadex G50 swollen in buffer with 0.02% (w/v) sodium azide (Pharmacia)
- 10mM Imidazole pH 6.1.
- 1mg/ml Blue Dextran

Method

Initially the size of the column to be used was determined from the concentration of protein to be applied. A G50 column will hold between 10 and 30mg protein per cm^2 of its cross-sectional area. Knowing the concentration of protein/ml of the sample to be applied, the cross sectional area can be determined and used to calculate the column volume, where $\pi r^2 h$ = column volume and πr^2 = cross sectional area. Taking into account that the sample volume must not exceed 5% of the column volume.

The G50 sephadex column was swollen in 10mM imidazole buffer pH6.1 for three hours before being poured into a glass column. Following which the void volume of the column was determined by the addition of 1ml of 1mg/ml solution of blue dextran, and washing with 10mM imidazole. Then the sample of calmodulin was applied and the column continually washed with 10mM imidazole buffer. 4ml fractions were collected throughout and the absorbance measured at 280nm.

2.3.2 Protein Determination

The method used is a variation of the method determined by Bradford (1978), using Coomassie Brilliant Blue G. The Coomassie blue exhibits an absorbance maxima at 465nm, which shifts to 595nm upon binding to proteins (Peterson 1983).

Materials

- 0.06% (w/v) Coomassie Brilliant Blue G250 in 0.3M Perchloric Acid.
- 0.9% NaCl (w/v).
- 1mg/ml Bovine Serum Albumin in water.

Method

1. A standard curve using bovine serum albumen was set up in 3ml cuvettes ($1cm^2$) in a range of 0 to 50μg protein, as shown in table 4.

2. Samples to be tested, that were opaque or part of a pellet, were first solubilized in 0.1M NaOH, microfuged and the supernatant removed for protein determination.

3. Between 1μl and 100μl of sample was placed in a 3ml ($1cm^2$) cuvette and sufficient saline added to bring the total volume to 1ml. Then 1ml coomassie solution was then added, and the absorbance measured at 620nm with the spectrophotometer being blanked against a solution of 1ml saline and 1ml Coomassie. Then the standard curve samples were measured before the test samples. The standard curve was plotted and the protein content calculated.

Standard Curve Experimental Setup		
Bovine Serum Albumin (µl=µg)	Volume of Saline (ml)	Volume of Coomassie (ml)
0	1,000	1
5	995	1
10	990	1
20	980	1
30	970	1
40	960	1
50	950	1

Table 4. Coomassie Blue Standard Curve

The table shows the volumes of the various reagents, to form the standard curve for Coomassie Blue analysis of protein concentration.

2.3.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Denaturing gels were used and the methods employed based on Hames and Rickwood (1990); Walker (1984); Laemmli (1970); Weber, Pringle and Osborn (1972).

Materials

- 30% (w/v)Acrylamide (Fisons A/0977)
- 2% (w/v) Bisacrylamide (N,N-methylene-bisacrylamide.
- 1M Tris-HCl pH8.7 and pH6.9
- 20% (w/v) sodium dodecyl sulphate (SDS)
- 10% (w/v) ammonium Persulphate
- N,N,N,N-Tetramethyl-ethylene -diamine (TEMED)
- x10 Running Buffer: 0.25M Tris, 1.92M Glycine, 1% (w/v) SDS pH8.3.
- Sample Buffer: 1.51% (w/v) Tris, 20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue.
- Molecular Weight Markers: (Sigma) SDS-7 Dalton Mark VII-L Molecular weight range of 14000 to 70,000; SDS-6 Dalton mark VI molecular weight range 14,000 to 70,000; SDS-6H molecular weight range 30,000 to 200,000.
- Staining Solution: 25% (w/v) Coomassie blue, 40% (v/v) methanol, 7% (v/v) acetic acid.
- Fixing Solution : 40% (v/v) Methanol , 7% (v/v) acetic acid .
- Anti-Cracking Buffer: 1% (v/v) glycerol, 10% (v/v) acetic acid

Method

1. The percentage gel required for the resolving gel was prepared using the quantities of 30% (w/v) acrylamide, 25(w/v) bisacrylamide, 1M Tris and water as detailed in Table 5. These solutions were mixed and degassed before the addition, of the specified quantities, of TEMED or ammonium sulphate. It was then further mixed and poured between the glass plates of the gel cast. See Figure 22. The gel

mixture was then overlaid with a small quantity of 0.1% (w/v) SDS and left to stand at room temperature until the gel had polymerised. After which the stacking gel was prepared as per table 5. omitting the TEMED and ammonium persulphate until it had been degassed. Following this the gel overlay was removed from the polymerised gel, the gel surface washed with double distilled water, and the stacking gel quickly poured. A comb giving the desired number of wells was then swiftly and carefully inserted before the gel was left to polymerise. Once set the gel was placed in the gel running tank with the top and bottom edges immersed in x1 running buffer (100ml 10x running buffer and 900ml ddw). Any air bubbles that formed in-between the plates were carefully removed without disturbing the gel. Figure 22.

In general an ATTO SJ-106059 gel electrophoresis system was used but occasionally a BioRad gel system was used. The principle difference between the two is that the BioRad system is cooled with cold tap water whereas the ATTO system is not.

2. During this time the samples (from sections 2.3.1 and 2.3.7.) for gel analysis were prepared as follows. If the protein samples contained potassium or greater than 0.2M salt, they were dialysed against 0.1% (w/v) NaCl for 1 to 2 hours, before being processed for electrophoresis. The samples and molecular weight markers were mixed with an equal volume of x2 sample buffer and boiled for 3 minutes. After which they were cooled, and if necessary microfuged for 1 minute to remove any precipitated protein. The samples were then loaded into the wells using a Gilson pipette or a Hamilton syringe and the gel run either overnight at 10mA, 40V or for approximately 4 hours at 150V, 10v/cm^2. It was run until the

Gel composition (% acrylamide:mg bisacrylamide/ ml)								
Resolving Gel	5% :2.6	7.5% :1.9	10% :1.3	12.5% :1	15% :0.86	17.5%	20%	Stacking Gel
30% Acrylamide (ml)	5	7.5	10	12.5	15	17.5	20	1.2
2% Bisacryl -amide (ml)	3.9	2.9	2	1.5	1.3	1.1	1	0.07
1M Tris pH 8.7 (ml)	11.2	11.2	11.2	11.2	11.2	11.2	11.2	1.25 (pH 6.9)
20% SDS (ml)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.05
H_2O (ml)	9.45	8.25	6.65	4.65	2.35	-	-	6.3
TEMED (µl)	25	25	25	25	25	25	25	25
10% Ammonium persulphate (µl)	100	100	100	100	100	100	100	100

Table 5 Gel composition for SDS-PAGE

The table shows the quantities required of the various gel components to prepare different percentage gels (Maniatis, Fritsch & Sambrook 1982).

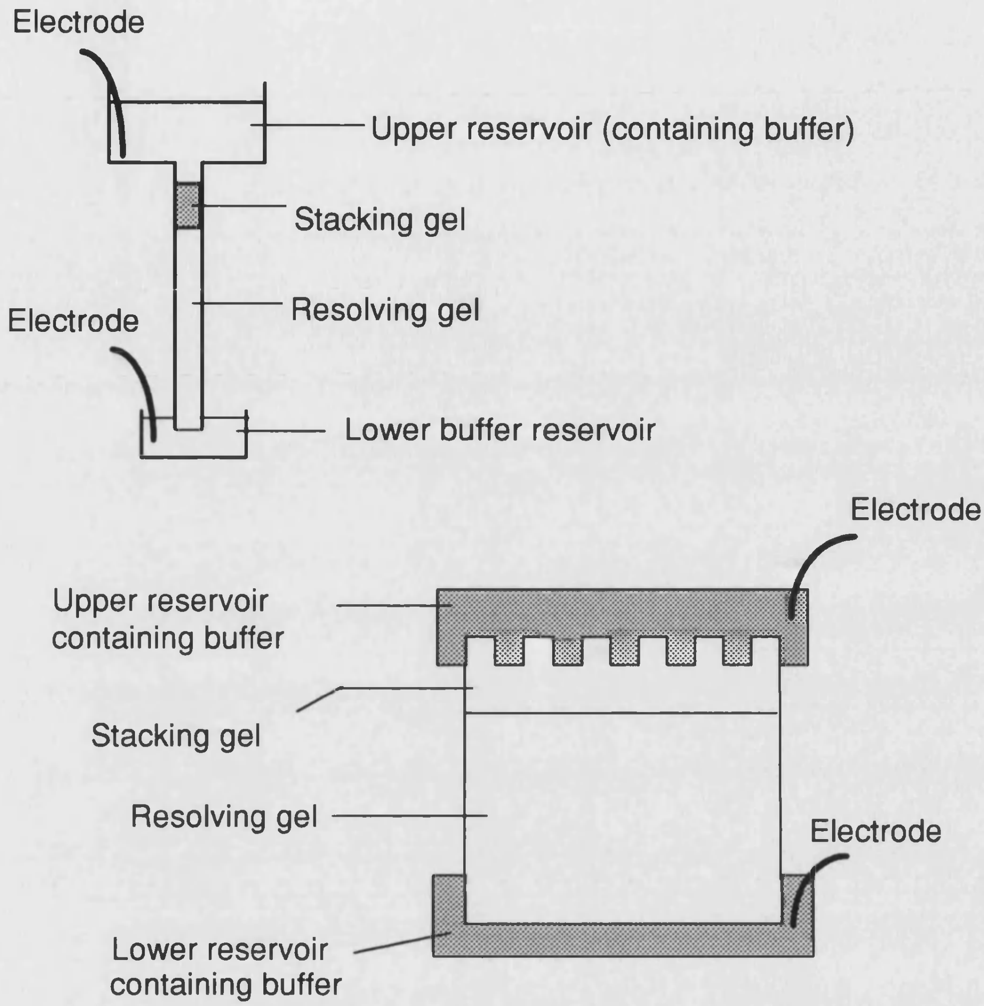


Figure 22. SDS PAGE Diagrammatic Representation Of Equipment

The diagram shows a side and front view of a polyacrylamide gel electrophoresis set up. The resolving gel is poured between two glass plates, which are held in place by clamps. Once set the stacking gel is poured on top and the comb inserted. When this has also polymerized the comb is removed and the samples loaded into the wells. The gel is then ready to run (Hames & Rickwood 1985).

marker dye was within 1-2 cm from the base of the gel plates.

3. The gel was then carefully removed and placed in fixing solution. Following which it was placed on a shaking platform and left for 24 hours with a few changes of fixing solution. After this the gel was left in staining solution for 24 hours with continual shaking. It was then destained with several changes of fixing solution over 48 hours whilst being gently shaken. Finally it was placed in anti cracking solution for 30 minutes before being either transferred onto Whatman 3MM filter paper and dried in an LKB Slab gel drier or sealed in a bag.

2.3.4 Phosphodiesterase Assay

This assay was used to test the biological activity of calmodulin and is based on the methods devised by Wallace, Tallant and Cheung (1983); and Means et al. (1991). Phosphodiesterase is regulated by calmodulin as described in the introduction (section 1.1.1.1.4), stimulating the enzyme's action up to 50 fold. The principle of the reaction is shown again below:

PDE (+ Calmodulin) + c3,5-AMP ----> 5'AMP

If this reaction is coupled to another reaction the 5'AMP can be converted into adenosine and free phosphate. Thus:-

5'AMP + alkaline phosphatase-----> adenosine + Pi.

This coupled reaction can then be used to test the biological activity of calmodulin samples as the free phosphate levels or the free adenosine levels can be measured. Because the methods for measuring phosphate are not very sensitive and not particularly accurate, measurement of adenosine is generally favoured. The rate of hydrolysis of cAMP can be quantified by using tritiated cAMP, which after cleavage will yield tritiated adenosine that can be measured in a scintillation counter.

Materials

- c3,5-AMP
- c-^{3}H-3,5-AMP (ICN Flow 24011 sp.ac.15-30 Ci/mol).
- Phosphodiesterase 3,5-Cyclic Nucleotide (Sigma P9529. 1 unit hydrolyzes 1μmol 3,5-cAMP to 5'AMP /min)
- Calmodulin (Sigma P2277)
- 2,8-^{3}H-Adenosine (Amersham, 20Ci/mmol)
- Isolated Calmodulin from either tapeworm or pig thymus (section 2.3.1)
- Reaction Buffer:- 50mM MOPS pH7.0, 12mM Magnesium Acetate, 20mM 2-Mercaptoethanol, 0.4mM $CaCl_2$. In some experiments Tris buffer was substituted for MOPS.
- AG1-X8 Ion-Exchange Resin (Biorad) 30% (w/v) in double distilled water.
- Alkaline Phosphatase Type II bacterial (Sigma P4252. 1 unit will hydrolyse 1μmol para-nitro-phosphate/min at pH 10.4 at 37 °C)
- Optiphase scintillation fluid (LKB)
- Stop Buffer:- 20mM EDTA, 10mM cAMP

Method

1. To a series of eppendorfs 50μl reaction buffer, 100μl PDE equivalent to 5mU activity and 30μl of double glass distilled water, commercial calmodulin or sample, were added making a total reaction volume of 200μl. This was pre-incubated at 30°C, for 10 minutes, before the addition of 20μl ^{3}H-cAMP equivalent to 0.02μCi, which should yield approximately 20,000cpm, to initiate the reaction. The mixture was incubated for varying lengths of time, depending on the purpose of the experiment, at 30 °C and was terminated either by boiling for 1 minute or by the addition of 200μl of stop buffer.

2. Following this to each tube 10µl alkaline phosphatase (19U) was added before being incubated for 15 minutes at 30°C. Then 1ml of AG1-X8 resin was added to each tube and the mixture vortexed twice with a 5 minute interval. Liberated ^{3}H-adenosine will remain free in solution whilst unreacted ^{3}H-c-3'5'-AMP will adsorb to the AG1-X8 resin.

3. After this, they were microfuged for 5 minutes and 500µl of the supernatant removed and mixed with 5ml of Optiphase.

The binding efficiency of the AG1-X8 resin, was checked by applying 20µl ^{3}H-cAMP to 1ml of AG1-X8, which was mixed, microfuged, the supernatant removed and added to 5ml of scintillent. To the resin 1ml 1M acetic acid was added to remove the bound ^{3}H-cAMP. It was mixed, centrifuged and the supernatant mixed with 5ml Optiphase. This procedure was repeated using ^{3}H-adenosine in place of the ^{3}H-cAMP. However, the ^{3}H-adenosine would not be expected to adsorb to the resin.

The total counts were determined by mixing 20µl ^{3}H-cAMP with 5ml Optiphase and the background determined by mixing 20µl water with 5ml Optiphase.

All the samples and controls were then vortexed before counting on an LKB TM1600 scintillation counter. From the results obtained the number of moles of cAMP hydrolysed were calculated.

2.3.5 Thin Layer chromatography

This was used to check the purity and stability of the ^{3}H-cAMP being used in the phosphodiesterase experiment (Randerath & Randerath 1967).

Materials

- TLC Plates: CEI Cel PEI cellulose
- 0.3M LiCl Buffer
- Butanol Running Buffer:- butanol: methanol: water: ammonia in a ratio of 60:20:20:1.
- 1mM Adenosine
- 1mM cAMP
- ^{3}H-cAMP (IGN-Flow)
- 0.5M Perchloric Acid
- Optiphase (LKB)

Method

1. 10µl to 20µl of the adenosine, cAMP and ^{3}H-cAMP were loaded onto a prewashed TLC plate, 2cm from its base as follows. 5µl of sample was streaked in a line (1.5cm long) using a 5µl capillary tube, and dried with a hairdryer before the next sample was applied. Once all the sample had been loaded the plate was placed into a glass chamber, with the base of the TLC plate immersed in butanol running buffer. The tank was covered and allowed to stand until the solvent front had reached the top of the plate. It was then removed and dried overnight, before being transferred to a chamber containing some LiCl buffer.

   Again it was then run until the solvent front reached the top of the plate. The plate was then dried and viewed with a UV lamp and the positions of the samples marked in pencil.
2. Each lane was then carefully cut either into 0.5cm^2 or 1cm^2 squares, maintaining the order from one end of the plate to the other and the cellulose scraped off and

placed into a scintillation vial. To which between 200 and 400µl 0.5M perchloric acid was added before the vials (with caps loosely on) were heated in an oven at 90°C for 30 minutes. They were then cooled and if the vial contents were dry, 200µl double glass distilled water was added before addition of 2ml Optiphase.

3. The vials were then vortexed before being counted with in a LKB TM1600 scintillation counter. The results were plotted, and the position of the nucleotides determined.

2.3.6 Phosphodiesterase Calmodulin Sensitivity Experiment.

An experiment was designed to test the stability of phosphodiesterase as manufacturer's have reported that phosphodiesterase, once prepared in solution, becomes unstable. The result of which is that over time the basal activity of the enzyme increases with a concomitant decrease in its sensitivity to calmodulin. To test this an experiment was designed using calmodulin-agarose.

Materials

- PDE Reaction Buffer:-40mM Tris pH8, 3mM $MgSO_4$, 50µM $CaCl_2$.
- Calmodulin Agarose (Sigma)
- Application Buffer:- 40mM Tris pH 7.5, 50mM NaCl, 1.5mM $CaCl_2$, 3mM $MgCl_2$, and 0.1mM dithiothreitol.
- Elution Buffer:- 40mM Tris-HCl pH 7.5, 50mM or 200mM NaCl, 3mM EGTA, 1mM $MgCl_2$, and 0.1mM dithiothreitol.
- Regeneration Buffer:- 40mM Tris-HCl pH 7.5, 3mM EGTA, 1M NaCl, 0.2% (w/v) sodium azide.
- Phosphodiesterase:- 1U/ml in application buffer (Sigma P9529)
- Alkaline Phosphatase:- 0.1U/ul in 0.1% bovine serum albumin. (Sigma P4252 Type II bacterial)
- Dowex Ion-Exchange Resin AG X18 (BioRad) prepared as a 33% (w/v) mixture.

- Optiphase scintillation fluid (LKB)

Method

1. Two 1ml calmodulin agarose columns were prepared, and pre-equilibriated with application buffer, and used for alternate time points throughout the experiment. The phosphodiesterase was prepared and at hourly intervals for 12 hours and at 24 hours 75µl (75mU) phosphodiesterase was added to a pre-equilibriated column, which was run dropwise, as follows. The column was washed with 5ml application buffer, followed by 5ml elution buffer containing 50mM NaCl and finally 5ml of elution buffer containing 200mM NaCl. The column was then regenerated using 5ml regeneration buffer, and equilibriated with application buffer ready for its next run. 500µl fractions were collected throughout in eppendorfs.
2. To each fraction collected, 70µl phosphodiesterase reaction buffer was added together with 30µl ^{3}H-cAMP, before being incubated at 30°C for 1 hour. They were then stored at room temperature until all time points had been applied to the columns.
3. Following this to each sample 10µl of alkaline phosphatase was added. The samples were then incubated at 30°C for 1 hour, after which 1ml of Dowex AG1-X8 resin was added. The samples were vortexed twice with a 5 minute interval before being microfuged for 5 minutes. 500µl of the supernatant was removed and mixed with 5ml Optiphase in a scintillation vial. Vials were also prepared to determine the binding efficiency of the resin and the total counts as

described in section 2.3.4. Each sample was then counted for 2 minutes on a LKB TM1600 scintillation counter.

2.3.7 Isolation of Calmodulin Binding Proteins

Affinity chromatography was used to isolate the calmodulin binding proteins from Hymenolepis diminuta.

Materials

- Application Buffer:- 40mM Tris pH 7.5, 50mM NaCl, 3mM $MgCl_2$, and 0.1mM dithiothreitol.
- Application Buffer solutions containing either 1.5mM $CaCl_2$ or 3mM EGTA.
- Elution Buffer:- 40mM Tris-HCl pH 7.5, 3mM EGTA, 1mM $MgCl_2$, and 0.1mM dithiothreitol.
- Elution Buffer solutions containing either 50mM or 200mM NaCl.
- Regeneration Buffer: 40mM Tris-HCl pH 7.5, 3mM EGTA, 1M NaCl, 0.2% (w/v) sodium azide.

Method

1. A 5ml calmodulin-agarose column was prepared and pre-equilibriated with application buffer. If the aim was to isolate calcium dependent calmodulin binding proteins then application buffer containing 1.5mM $CaCl_2$ was used. But for calcium independent calmodulin binding protein's application buffer containing 3mM EGTA was used.
2. 10g of frozen tapeworms were homogenised with a Sorval omnimix in 20ml of application buffer containing either 1.5mM $CaCl_2$ or 3mM EGTA depending on whether calcium dependent calmodulin binding proteins or calcium independent binding proteins were required. The homogenate was filtered through four layers of muslin cloth, before centrifugation at 36,000g, at 4°C, for 30 minutes. The

supernatant was removed and filtered through four layers of muslin cloth, before application to the agarose column, at a rate of 0.5ml per minute.

3. The column was washed with application buffer, until the absorbance at 280nm was less than 0.05 to remove any unbound protein. The calmodulin binding proteins were eluted with a maximum of 5 column volumes of elution buffer containing 50mM NaCl. Finally to remove any tightly bound proteins 5 column volumes of elution buffer containing 200mM NaCl was applied. The column was regenerated with 10 column volumes of regeneration buffer and stored at 4°C. Throughout fractions were collected and the absorbance measured at 280nm. Peaks of absorbance were pooled and their protein content determined by Coomassie (section 2.3.2.) and if necessary concentrated by Amicon centricon as per manufacturers' instructions. The fractions were then analysed by polyacrylamide gel electrophoresis (section 2.3.3).

2.3.8 ELISA

An ELISA (enzyme linked immunosorbant assay) was performed to check that samples thought to be calmodulin, from section 2.3.1., were calmodulin and had retained their antigenicity.

Materials

- Coating Buffer: 0.12% (W/V) NA_2CO_3, 0.3% (W/V) $NAHCO_3$ PH9.6
- Stock Substrate: Tetramethyl -benzidine (TMB) 10mg/ml in dimethylsulphoxide.
- Citrate Buffer: 0.63% (w/v) citrate pH6, 13.6% (w/v) sodium acetate
- Sample containing calmodulin from section 2.3.1.
- Calmodulin (Sigma)

- Working Substrate Solution:- The citrate buffer was diluted 1:20 in double distilled water pH6. Then, to 49.5ml buffer, 0.5ml stock solution of TMB and 10μl of 30% (v/v) hydrogen peroxide were added.
- Phosphate Buffered Saline (PBS):- 0.7% (w/v) NaCl, 0.34% (w/v) Na_2HPO_4, 0.08% (w/v) KH_2PO_4.
- 0.1% (v/v) Tween-20 (Polyoxethylene sorbitan monolaurate) in PBS.
- Anti-Sheep IgG (whole molecule) peroxidase conjugated (Sigma A-3415 titre 1:14,000 by direct ELISA)
- Anti-Calmodulin (sheep) (Calbiochem 208692, approx. working dilution 1:50,000 in ELISA and dot blot analysis. 50ng calmodulin should be detectable in ELISA or dot blot.)
- 1.84M H_2SO_4
- Anti-Calmodulin (goat) (Sigma)
- Anti-Goat IgG conjugated with peroxidase (Sigma)

Method

1. A 96 well microtitre plate was pre-coated over night at 4°C with 100μl antigen (sample calmodulin or manufacturers), suitably diluted to 10μg/ml in coating buffer. Following this the plates were washed three times with 0.1% (w/v) TWEEN in PBS.
2. The anti-calmodulin was prepared as a 1-100 dilution of the stock antibody in the 0.1% (w/v) TWEEN in PBS and then applied to the wells pre-coated with antigen, in serial dilution. The plate was then incubated for either $1^1/_2$ hours at 37°C or 2 to 3 hours at room temperature. After which it was washed four times with 0.1% (w/v) TWEEN in PBS.
3. To each well 100μl anti-sheep antibody (Ig peroxidase conjugated) was applied diluted 1-1000 in 0.1% (w/v) TWEEN in PBS and the plate left for 2 hours at

room temperature. The antibody was then removed by washing 3 times with 0.1% (w/v) TWEEN in PBS, followed by a further 2 washes with PBS.

4. After this, 100µl of working substrate solution was applied to each well and the plate left to stand for between 10 and 30 minutes at room temperature. Allowing sufficient time for the colour (blue) to develop by the interaction of the tetramethylbenzidine and the peroxidase conjugated antibody. The reaction was stopped by the addition of 50µl 1.84M H_2SO_4 per well which converts the blue colour to yellow.
5. The optical density was then measured at 450nm using a Multiskan MCC plate reader (Labsystems) and the results recorded.

2.3.9 Western Blot

A Western blot was used to help identify the protein that was reacting with the anticalmodulin in the ELISA. The method used is based on that of Towbin, Staehelin and Gordon (1979).

Materials

- Anti-Goat IgG conjugated with peroxidase. (Sigma)
- Sample containing calmodulin from section 2.3.1.
- Calmodulin (Sigma)
- Anti-Calmodulin (goat) (Sigma)
- Methyl Green (donated)
- Blot Buffer:- 25mM Tris, 192mM Glycine, 20 % (v/v) methanol pH 8.3 .
- Anti-Sheep IgG (whole molecule) peroxidase conjugated (Sigma A-3415 titre 1:14,000 by direct ELISA)
- Anti Calmodulin (sheep) (Calbiochem 208692, approx. working dilution 1:50,000 in ELISA and dot blot analysis. 50ng calmodulin should be detectable in ELISA or dot blot.

- Phosphate Buffered Saline (PBS):- 0.7% (w/v) NaCl, 0.34% (w/v) Na_2HPO_4, 0.08% KH_2PO_4.
- 1% (w/v) casein in PBS
- 0.1% (v/v) TWEEN in PBS
- Stock Substrate Solution:- 4-chloro-1-napthol 3mg/ml in methanol.
- Working Solution Of Substrate:- 2ml stock solution, 10 ml PBS, 5μl 30% (v/v) hydrogen peroxide.

Method

1. Samples (from section 2.3.1) to be analysed were subjected to polyacrylamide gel electrophoresis as previously described in section 2.3.3. The only difference being that while the gel was running, two sets of 5μl methylene green were loaded into the wells and allowed to run through the gel. The purpose of this was to allow identification of the gel lanes, and the direction the lanes have run.
2. Once electrophoresis was completed, the gel was removed from the glass plates and cut in two. One half was stained with Coomassie blue and the other duplicate half was set up in the blot apparatus (BioRad) as shown in Figure 23. The blot tank was filled with blot buffer and the blot performed at 30V (0.1amp) overnight at 4°C.

   After which the orientation of the nitrocellulose onto which the proteins had been transferred, was carefully marked before being cut into strips corresponding to the lanes of the gel. Each strip, representing one lane of the gel was divided in two. One half would be treated with both anti-calmodulin and anti-sheep (or goat) Ig

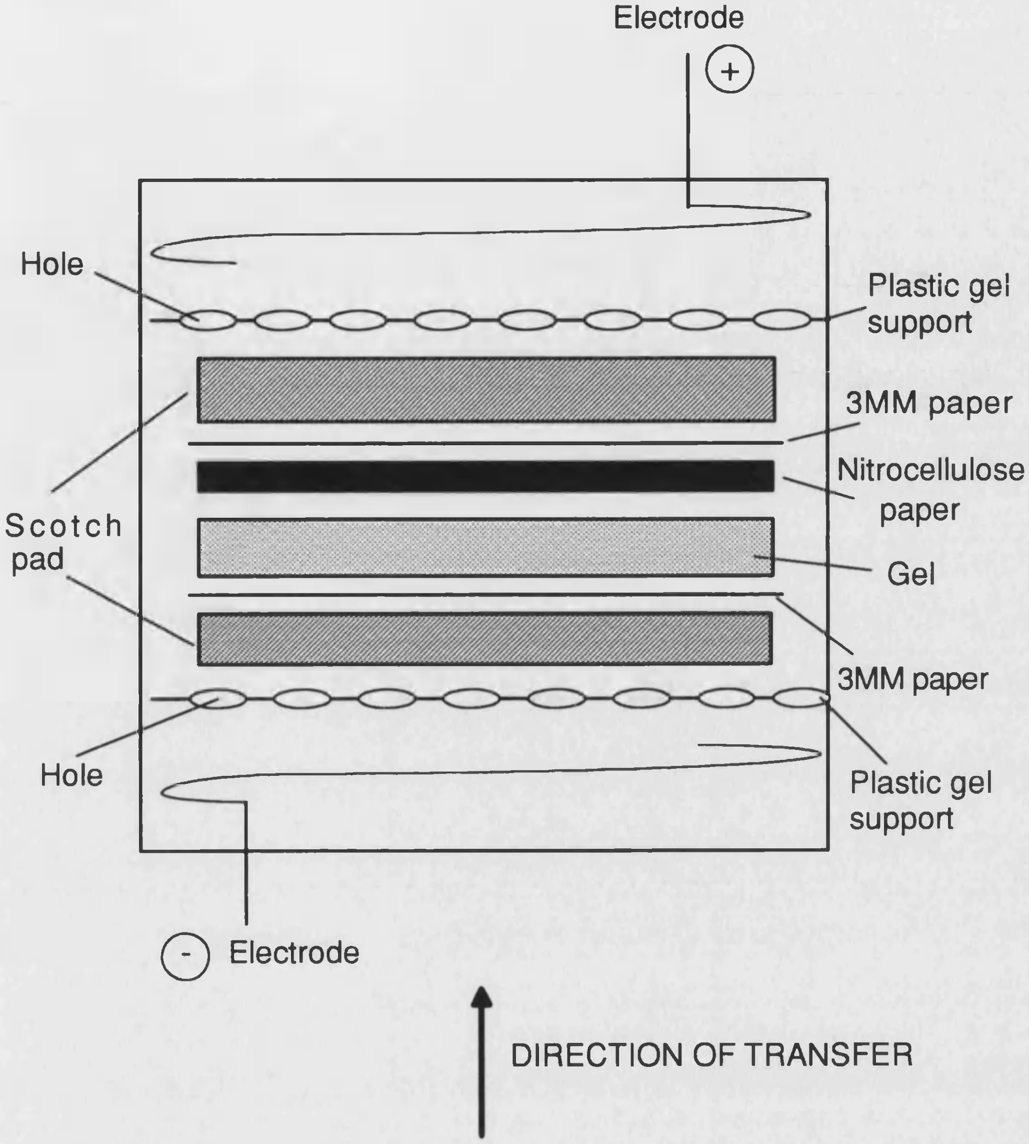


Figure 23. Diagram Showing Arrangement Of Western Blot

The diagram shows an aerial view of the western blot apparatus. The gel, nitro-cellulose, and filter paper are sandwiched between two scotch pads supported between two plastic supports. This 'sandwich' is placed within the tank, which is ≈30cm high and ≈15cm wide. The tank is filled with blot buffer and run overnight at 30V.

peroxidase conjugated; whilst the other half would serve as the control and would not be treated with anti-calmodulin.

3. The strips were placed into a mini incubation tray and each strip washed for 2x5 minutes with 4ml PBS, to remove the sodium dodecyl sulphate from the gel, and the buffer aspirated off. Following this any unbound sites were blocked by the incubation of each strip with 4ml 0.1% (w/v) casein in PBS for 1 hour, at room temperature, on a shaking platform. They were then washed twice with 0.1% (v/v) TWEEN in PBS (4ml/strip) for 2x5 minutes. The buffer being removed by aspiration each occasion.

4. After this to half of the strip's 4ml anti-calmodulin was applied, suitably diluted in 0.1% (v/v) TWEEN in PBS as determined from the ELISA in section 2.3.8. To the remaining half of the strip's, 4ml of 0.1% (v/v) TWEEN in PBS was applied. All the strips were incubated at room temperature, on a shaking platform, for 3 hours and then washed twice with 0.1% (v/v) TWEEN in PBS (4ml per strip) for 5 minutes each time. This will remove any unbound anti-calmodulin, where present.

5. To each strip, 4ml anti-sheep was added diluted 1-1000 in 0.1% (v/v) TWEEN in PBS. They were then left for 2 hours at room temperature before being washed three times with 4ml 0.1% (v/v) TWEEN and twice with 4ml PBS. On each occasion the strips were left for 5 minutes and the buffer then aspirated off.

6. Finally 4ml per strip of working substrate solution was applied and the strips allowed to stand for between 30 minutes and overnight, as necessary. Positive hybridisation of the antibody with a protein should result in a dark band on the nitro-cellulose strips, formed by the reaction of the second peroxidase conjugated

antibody and the substrate. Once dark bands were apparent the strips were washed with 4ml PBS per strip. They were then attached to a piece of Whatmans 3MM filter paper which was wrapped in foil and stored.

2.4 IMMUNOCYTOCHEMISTRY

Immunocytochemistry was performed to localise calmodulin in situ in the tapeworm tissue. Trial experiments were tried using immunofluoresence, before the more expensive immunogold experiments were performed.

2.4.1 Preparation of Tissue for Electron Microscopy

Two factors had to be offset in determining the best fixing method for the tapeworm. The first is that the tissue processing had to be as rapid as possible to ensure maximum retention of antigen, in this case calmodulin. The second factor was to ensure that the tissue was sufficiently well preserved that the structure would still be identifiable.

Materials

- Minimal Essential Media (MEM):- 15mM HEPES (N-2-hydroxyethyl -piperazine-N-2-ethanesulphonic acid), 8.7% (v/v) MEM concentrate (Flow Labs), 8.7% (v/v) foetal calf serum (Globe Farm), 173 IU/ml Penicillin , 17% (w/v) streptomycin, 1.7mM glutamine, 1.7% (v/v) x50 amino acids for MEM (Flow Labs)
- Milonig Phosphate Buffer:- 0.02M $NaH_2PO_4.H_2O$, 0.126M $Na_2HPO_4.7H_2O$ pH7.4. Osmolarity = 320.
- 0.1M Sorensons Phosphate Buffer:- Made from 0.2M Na_2HPO_4, 0.2M NaH_2PO_4 stock solutions. At pH 7.4, this required 40.5ml of Na_2HPO_4, and 9.5ml of NaH_2PO_4. (226mOsm)
- 10% (w/v) Stock Formaldehyde
- 4% (v/v) Formaldehyde in Milonig buffer
- 1% (v/v) Gluteraldehyde, 4% (w/v) sucrose in 0.1M Sorensons phosphate buffer.
- 5.5% (v/v) Gluteraldehyde, 0.9% (v/v) Osmium Tetroxide, 50mM Sorensons phosphate buffer.
- 1% (w/v) Tannic Acid in 0.1M Sorensons buffer

- 10%, 30%, 50%, 70%, and 100% (v/v) Ethanol.
- LR-White Resin (Agar Scientific Ltd.)
- Plastic Truffs (manufactured by LKB, supplied by Agar Aids Ltd.)
- Copper and Nickel electron microscope grids.

Method

1. Tapeworms were isolated from the rat intestine (section 1.1.) and placed in minimal essential medium until required. Each tapeworm was then divided into five different regions: head, neck, upper-middle, middle and tail. A minimum of three segments from each region were placed into a "basket" (a small plastic cylinder with nylon mesh covering one end) and for each process tried all five regions were processed. All processes prior to polymerization took place in the baskets and were performed on ice unless otherwise stated. A generalised scheme of the preparation process is shown in figure 24.
2. Several fixatives were tried all of ≈300mOsm on different batches of tapeworms. These included: 4% (v/v) formaldehyde, 1% (v/v) gluteraldehyde and a mixture of 5.5% (v/v) gluteraldehyde, 0.9% (v/v) osmium tetroxide, 50mM Sorensons phosphate buffer. In each case the tapeworm segments were fixed for 1 hour. After fixing the samples were rinsed with 3 changes of 0.1M Sorensons phosphate buffer containing 4% (w/v) sucrose for 10 to 15 minutes. Then the samples that were treated with osmium tetroxide were divided in half. Half were incubated with 1% (w/v) Tannic acid, 0.1M Sorensons buffer for 30 minutes before dehydration; whilst the remaining half were dehydrated immediately.

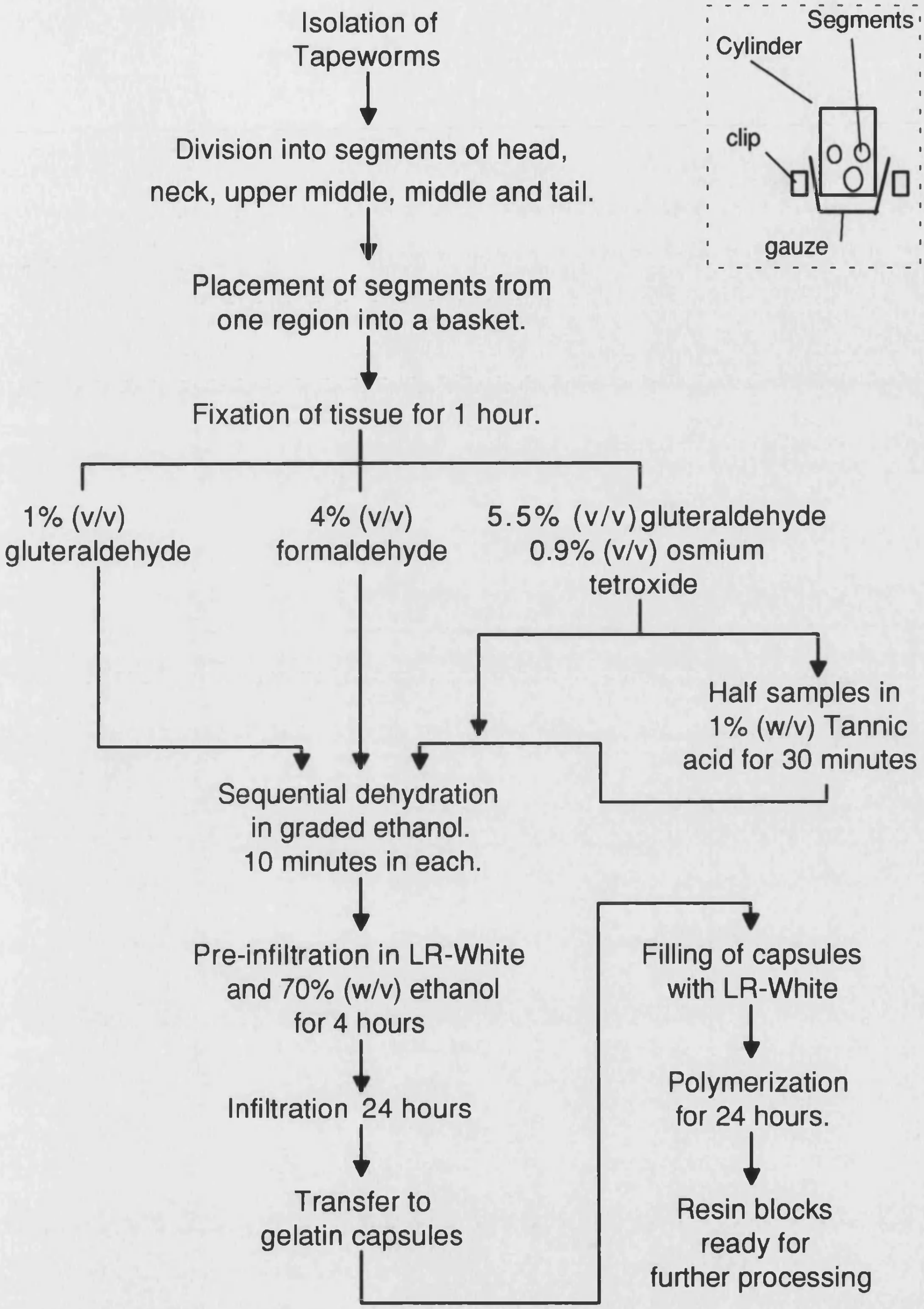


Figure 24. Scheme for Preparation of Tissue for Immunocytochemistry.

Tissue is fixed, dehydrated, infiltrated with resin and polymerised into blocks. These are then processed further for sectioning. Inset shows diagram of a 'basket'.

2. After fixation all the samples were dehydrated in a series of graded ethanol from 10 to 100% (v/v) with 10 minutes in each grade prior to being pre-infiltrated with a mixture of LR-White and 70% (v/v) ethanol in a ratio of 2:1 for four hours at 4°C. Following which the samples were infiltrated with LR-White for 24 hours, at 4°C, on a rotating platform, with one change of resin after the first 1 to 3 hours. Then each segment was carefully removed and placed into a pre-warmed (50°C) gelatin capsules. The capsule was labelled with a piece of marked paper before being filled with fresh LR-White and sealed and the resin polymerised at 50°C for 24 hours.

4. The resulting blocks of resin were prepared for sectioning with a Reichert Om U3 ultramicrotome using glass knives. Semi-thin (0.75microns) sections were cut first and stained with toluidine blue, as described in section 2.4.2., before being viewed under a light microscope. If infiltration of the resin was satisfactory the blocks were processed further. Initially more semi-thin sections were cut and processed for immunofluorescence, section 2.4.3. As the work advanced thin sections (90nm) were then cut from the blocks and floated off onto a pool of filtered distilled water contained in plastic truffs. The thin sections were collected on either copper or nickel grids, which were blotted dry and kept for immunogold experiments (section 2.4.4.).

2.4.2 Toluidine Blue Staining of Sections

To check the tissue preservation and the infiltration of the resin slides were prepared of semi-thin sections.

Materials

- Toluidine Blue Stain
- BDH Mounting Fluid

Method

Semi-thin sections, from section 2.4.1., were placed onto clean slides and dried on a heating block for several minutes. Then sufficient toluidine blue to cover the sections, was syringed onto the slide being passed through a 0.3μm filter first. The slide was then placed on a heating block, for a few minutes, until the outer edges of the toluidine blue turned golden green. At which point the slide was removed and the toluidine blue rinsed off with double distilled water. The slide was returned to the heating block to dry. Once dry a small drop of BDH Mounting fluid was put onto the slide, and a coverslip placed on top. It was then ready to view with a light microscope.

2.4.3 Immunofluorescence Studies

Immunoflourescence studies were performed on semi-thin sections and were used to assess the viability of using immunocytochemistry to localise calmodulin. The method used is a post embedding technique and based on that of Slot and Gauze (1984).

Materials

- Anti-Calmodulin (sheep) (Calbiochem)
- Anti-Sheep IgG conjugated with FITC (flouroscein isothiocyanate) fluor. (Sigma)
- Phosphate buffered saline (PBS):- 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4 pH 7.2, 0.15M NaCl
- 1% (w/v) Bovine serum albumin in PBS

- 0.02M glycine in PBS
- Vectorsheild mounting fluid. (donated).

Method

1. Semi-thin sections, from section 2.4.1., were prepared and dried onto glass slides. In an attempt to de-lipify the tissue and reduce its natural fluorescence some of the slides were washed with acetone. The slides were then placed in a humidity chamber, in which all subsequent took place.

The slides were covered with the following series of blocking agents: 1% (w/v) bovine serum albumin in PBS for 5 minutes, 1% (w/v) gelatin for 10 minutes and two changes of 0.02M glycine in PBS for 5 minutes each. The slides were then rinsed with 0.1% (w/v) bovine serum albumin in PBS.

2. The anti-calmodulin was applied as a 1-15 dilution made with 0.1% (w/v) bovine serum albumin in PBS. Control slides were covered with either anti-calmodulin pre-sorbed with calmodulin or just 0.1% (w/v) bovine serum albumin in PBS. The slides were then left for 1 hour at room temperature and then rinsed with 5x 1 minute washes with 0.1% (w/v) bovine serum albumin in PBS. Following this the anti-sheep-antibody conjugated with fluor was added to each slide at a dilution of 1-25 made with 0.1% (w/v) bovine serum albumin in PBS. The slides were then left to stand for 1 hour at room temperature after which they were rinsed with 3x1 min washes with PBS. Then they were dried and approximately 10µl of Vectorsheild mounting fluid was applied.

3. The slides were then viewed with an inverted fluorescent microscope.

2.4.4 Immunogold studies

Materials

All solutions were freshly made immediately prior to use and filter sterilized.

- Tris Buffered Saline (TBS):- 50mM Tris-HCl pH8.2, 0.9% (w/v) NaCl
- TBS containing 1% (w/v) bovine serum albumin
- TBS, containing 1% (w/v) gelatin
- TBS containing 0.02M glycine
- Phosphate Buffered Saline (PBS):-2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM Na_2HPO_4 pH 7.2, 0.15M NaCl
- PBS containing 1% (w/v) bovine serum albumin
- PBS containing 1% (w/v) gelatin
- PBS containing 0.02M glycine
- Anti-Calmodulin (goat) (Sigma)
- Anti-Goat IgG conjugated with 10nm colloidal gold.(Sigma)

Method

The following treatment was performed on thin sections on either nickel or copper grids from section 2.4.1. All stages were carried out in a humidity chamber, with 20μl droplets of a solution being placed on a sheet of dental wax. Grids were then placed on these droplets upside down and transferred between one solution and the next ensuring that they did not dry out inbetween.

1. Initially the grids were blocked with the following series of solutions. 10 minutes in PBS containing 1% (w/v) bovine serum albumin followed by 15 minutes in PBS containing 1% (w/v) gelatin with one change of solution after 5 minutes. Finally they were placed in PBS containing 0.02M glycine for 10 minutes, with one change of solution after 5 minutes.

2. After this the grids were incubated for 1 hour with anti-calmodulin (IgG fraction) raised in goat diluted 1-10 or 1-5 with PBS containing 0.1% (w/v) bovine serum albumin. Then they were washed for 5x1 minutes with TBS containing 0.1% (w/v) bovine serum albumin.

3. Following this there was an 1 hour incubation with anti-goat IgG conjugated with colloidal gold diluted 1-10 or 1-5 with TBS containing 0.1% (w/v) bovine serum albumin. After which there were three 1 minute washes with 0.1% (w/v) bovine serum albumin in PBS; three 1 minutes in PBS and finally three 1 minute washes with double distilled water.

   With each experiment a control was included where the sections were only incubated with the second antibody, the first antibody (anti-calmodulin) being replaced by 0.1% (w/v) bovine serum albumin. An additional control was used to check the specificity of the anti-calmodulin, where the antibody was pre-sorbed with calmodulin before incubation with the grid.

4. After the immunostaining, the grids were counter-stained with uranyl acetate and lead citrate before being viewed with a Jeol JEM-1200 EX II transmission electron microscope.

In later experiments the PBS based solutions were replaced by TBS based solutions.

2.4.5 Counterstaining Protocol

Materials

- Reynolds Lead Citrate (Reynolds 1963)
- 2% (w/v) Uranyl Acetate
- Whatman No.54 Filter Paper
- 1M NaOH (BDH)

Method

After incubation with the antibodies, section 2.4.3., the grids needed to be counterstained. The method used, was that of Reynolds (1963).

The grids were incubated for 5 minutes in 100µl droplets of uranyl acetate, on a sheet of dental wax, in the dark. They were then washed 5 times with filtered double glass distilled water for 1 minute. They were then transferred to 100µl droplets of lead citrate, on a sheet of dental wax, in a petri dish, with pieces of filter paper on either side saturated with NaOH, for 5 minutes. The grids were then blotted dry with filter paper and were then ready for viewing with the JEOL 1200EXII transmission electron microscope.

3 RESULTS

The results obtained from the molecular biology, protein biochemistry and immunocytochemistry will be presented in their respective categories.

3.1 Molecular Biology

3.1.1 DNA Extraction Procedures

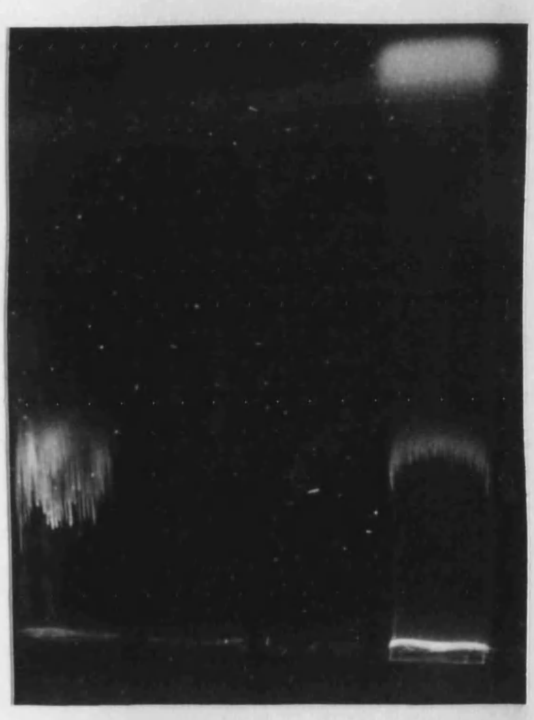
The purity of all the DNA samples obtained was determined by dividing the absorbance at 260nm by the absorbance at 280nm. Pure preparations of DNA and RNA have purity ratios of 1.8 and 2.0 however contamination with protein or phenol will considerably reduce this ratio (Maniatis, Fritsch & Sambrook 1989). The values obtained for the purity ratio ranged between 1.22 and 2.51. See Table 6. This also shows that the best method for isolating DNA was the CTAB method, as this consistently resulted in DNA of reasonable size, quality and concentration.

Figure 25 shows a sample of DNA extracted using both the proteinase K, phenol extraction method (section 2.2.1.1) and the CTAB method (section 2.2.1.2). Both samples show contamination in the well, which is probably either glycogen or protein. The heaviest contamination can be seen in the sample obtained using the proteinase K method. However, the DNA from both preparations appears to be between 20 and 30kb in size.

DNA Isolation Procedure	Purity Ratio of Isolated DNA	Concentration of DNA (mg/ml)	Size on Agarose gels.
Proteinase K and Phenol extraction (method 1. section 2.2.1.1.)	1.28	0.64	-
	1.6	1.55	-
	Mean = 1.41	Mean = 1.1	-
CTAB (method 2. section 2.2.1.2.)	2.75	0.11	23-30kb
	2.1	2.5	none visible
	2.4	1.88	none visible
	2.51	3.59	>20kb
	1.84	0.52	>20kb
	2.23	3.52	-
	1.25	9.5	-
	1.22	6.6	-
	Mean = 2.04	Mean = 3.53	-
Molecular Biosystems Kit (method 3. section 2.2.1.3.)	nd	none detected	-
Saponin/ Triton/ Chloroform (method 4. section 2.2.1.4.)	2.29	2.95	Sheared 1-10kb
	2.03	1.5	<6kb
	Mean = 2.16	Mean=2.23	-
Isolation of nuclei/ Proteinase K and Phenol chloroform extraction (Method 5. section 2.2.1.5.)	2.19	2.56	-
	1.6	3.24	largely RNA
	Mean = 1.89	Mean = 2.9	-

Table 6 Typical Values Obtained for Each of the DNA Isolation Procedures

For each sample of DNA that was isolated the absorbance was measured at 260nm and 280nm. From this the concentration and the purity of the DNA could be determined. Typical values obtained from each of the different extraction methods used are shown in the table above. It also shows how the DNA behaved on analysis by agarose gel electrophoresis.



CTAB DNA Proteinase K DNA

Figure 25. 0.3% Agarose Gel Showing Samples of Isolated DNA

Samples of DNA obtained using the CTAB and proteinase K methods (section 2.2.1.2. and 2.2.1.1.) were analysed by agarose gel electrophoresis, which showed that both samples were contaminated with either protein or more likely glycogen.

3.1.2 Digestion of Isolated DNA

The CTAB DNA preparations, containing good quality DNA, were used for digestion with restriction enzymes. The DNA was digested for 6 hours with EcoR1, BamHI, HindIII, PstI and a combination of EcoRI and BamHI as described in section 2.2.4. On 0.6% gel analysis it was found that EcoRI, BamHI, and PstI cut the DNA samples. See figure 26. As this was successful the gel was used for Southern blotting (section 2.2.3.). Unfortunately the resulting autoradiograph showed signs of non-specific binding of the oligonucleotide probe. Consequently the digestion and Southern blot were repeated. On this occasion the digestion was performed for 18 hours, to give a more digested sample containing a wider size range of DNA. See figure 27. Unfortunately, the background was again too high, showing that more stringent washing was required.

A Sau3A digest was also performed on a sample of CTAB DNA using various concentrations of Sau3A. This was to determine the optimum range for generating fragments of DNA of suitable length, for formation of a genomic library. It was found that using 0.125U and 0.062U of Sau3A produced the most satisfactory results. Once the quantity of Sau3A to be used had been determined the optimum time of digestion also had to be determined. Consequently a time course experiment was performed using 0.125U, 0.062U and 0.031U Sau3A. See figure 28. Fragments of digested DNA were visible after 5 minutes digestion when 0.125U Sau3A was used. When 0.62U Sau3A were used no fragments were visible until after 40 minutes of digestion. The same was true of the DNA incubated with 0.031U Sau3A only the bands were much fainter.

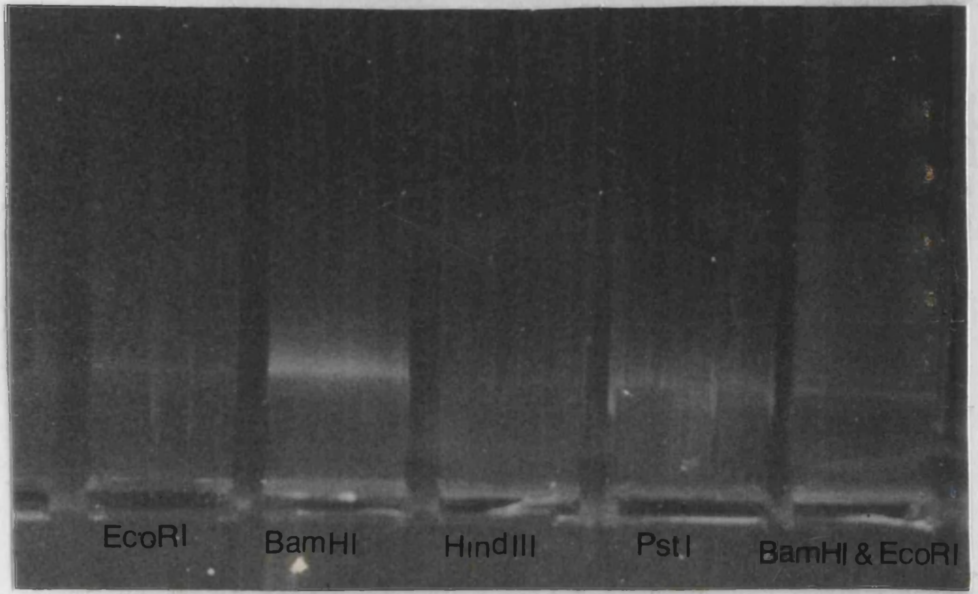


Figure 26. Restriction Digest of CTAB Isolated DNA.

Samples of tapeworm DNA were digested with EcoR1, BamHI, HindIII, PstI and a combination of either EcoRI and BamHI for 6 hours at 30°C.

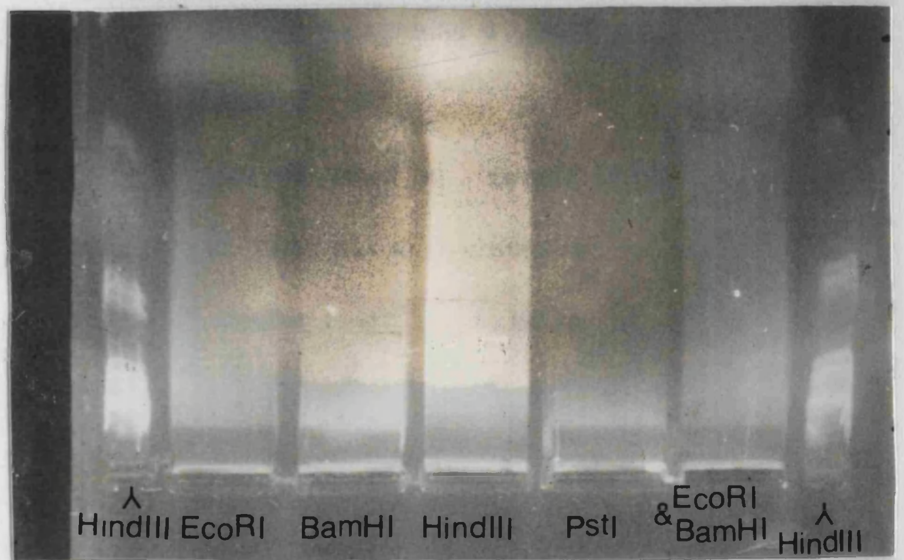


Figure 27. Restriction Digest of Tapeworm DNA Isolated Using the CTAB Method.

Samples of tapeworm DNA were treated with EcoR1, BamHI, HindIII, PstI and a combination of either EcoRI and BamHI for 18 hours at 30°C.

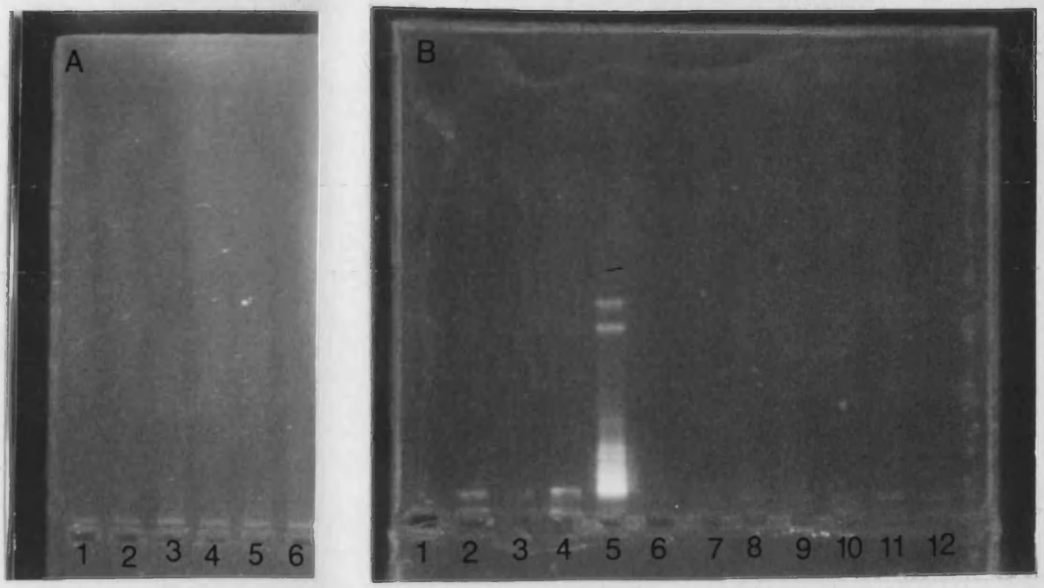


Figure 28. Time Course Digestion of Tapeworm DNA with Sau3A.

Figure A. DNA digested with 0.125U Sau3A. Lane 1=5 minutes, lane 2=10 minutes, lane 3=30 minutes, lane 4=30 minutes, lane 5= 40 minutes and lane 6= 50 minutes. It can be seen that fragments of digested DNA are visible after 5 minutes digestion with 0.125U Sau3A. **Figure B.** Lanes 1 to 4 show DNA digested with 0.062U Sau3A. Lane 1=30 minutes, lane 2=40 minutes, lane 3=50 minutes and lane 4=60 minutes. Lanes 6 to 12 show DNA digested with 0.031U Sau3A. Lane 6=5 minutes, lane 7=10 minutes, lane 8=15 minutes, lane 9=20 minutes, lane 10=30 minutes, lane 11=40 minutes and lane 12=50 minutes. Fragments are not visible until after 40 minutes incubation with 0.062U Sau3A. This is also true for DNA incubated with 0.031U Sau3A but the bands appear much fainter.

3.1.3 Amplification of DNA

3.1.3.1 Synthesis of Oligonucleotide Primers

The concentration of the synthesised 42 base oligonucleotide was 1.16mg/ml whilst the 39 base oligonucleotide had a concentration of 0.2mg/ml. From this it was deduced that both of the oligonucleotide probes had been incorrectly synthesised. This was checked by analysing the size of oligonucleotides by polyacrylamide gel electrophoresis (section 2.2.5.3.). From which it was evident that the 42 base primer consisted of oligonucleotides of 10, 13, 14, and 19 bases whereas the 39 base primer consisted predominantly of 5 base long oligonucleotides. See figure 29. The reason for the incorrect synthesis was found to be due to one of the bottles containing the chemicals for synthesis being incorrectly attached to the synthesiser combined with some blocked lines. Consequently the oligonucleotides were re-synthesised. The re-synthesised 39 based primer had a concentration of 2.7mg/ml, whilst the 42 based oligonucleotide was 2.8mg/ml. When their size was checked by polyacrylamide gel electrophoresis it was found that both primers were of the correct length. See figure 30.

3.1.3.2 PCR

When an annealing temperature of either 52°C or 42°C with either 10mM, 20mM or 30mM $MgCl_2$ was used with the isolated DNA, no PCR product formed. However, at an annealing temperature of 37°C, with 10mM $MgCl_2$, and to a lesser extent 20mM $MgCl_2$, PCR product was formed which was about 2kb as determined by 1% agarose gel electrophoresis. This gel was then Southern blotted and probed with the 50 base

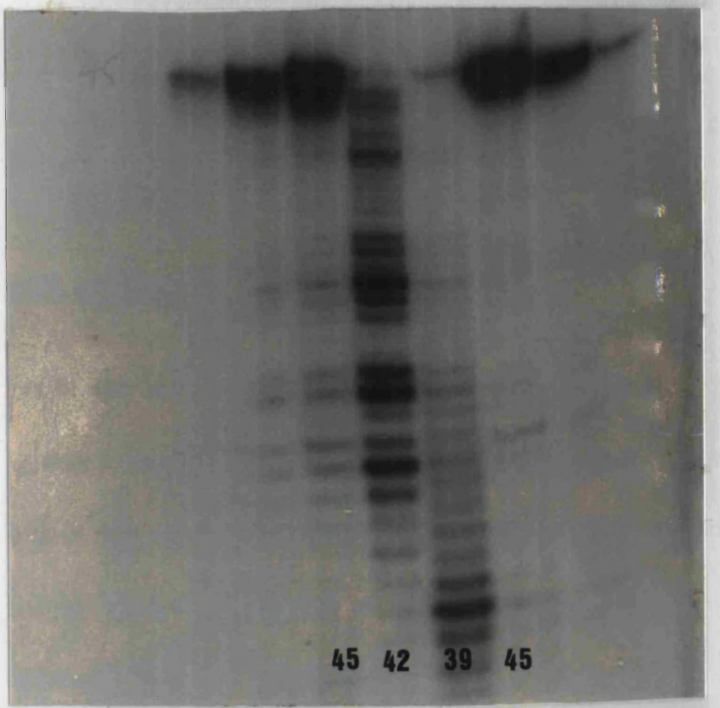


Figure 29. Sizing of Oligonucleotide Primers from the First Synthesis.

Photograph of the autoradiograph of the polyacrylamide gel used to size the oligonucleotide primers. The 42 base primer consists of oligonucleotides of 10, 13, 14, and 19 bases whilst the 39 base primer consists predominantly of 5 bases.

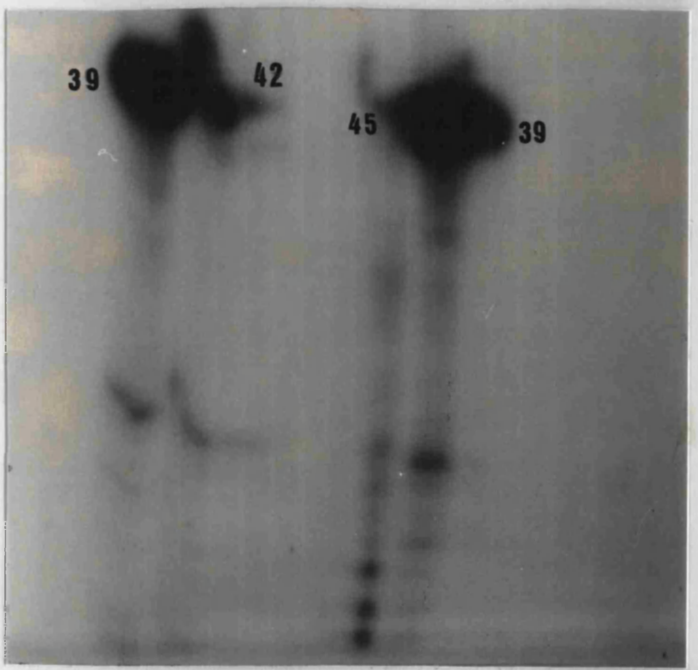


Figure 30. Sizing of Oligonucleotide Primers From Resynthesis.

The oligonucleotides have been synthesised correctly and consist of 42 and 39 oligonucleotides respectively.

oligonucleotide probe and positive hybridization occurred. See figure 31. This indicated that the calmodulin gene was present in the PCR DNA (Brook, Branford-White & Whish 1992). Consequently it would be possible to insert this into a vector and amplify it so that the calmodulin gene or part of it could be sequenced.

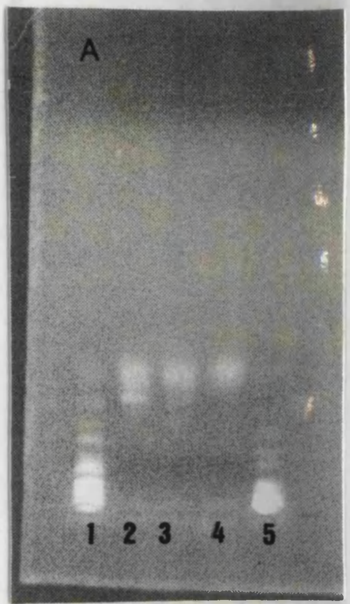
3.1.4 Cloning of Selected DNA

3.1.4.1 Ligation and transformation of PCR product into vector

When M13 was used as vector, a very low transformation rate was obtained. When pUC was used the transformation rate was higher. As the transformation rate was so low, hexamine cobalt was added to the ligation mix, in case the problem was due to ineffective ligation of PCR DNA into the vector. Unfortunately this did not appear to enhance the rate of transformation.

To check that the problem was not with the E.coli cells, a growth curve was prepared. A culture of E.coli (TG1's) was grown in either Luria Bertini broth or double yeast tryptone broth at 37°C and samples taken every 20 minutes. The optical density was measured at 550nm and the results plotted. The result of this can be seen in figure 32. It can be seen that the growth of the E.coli is normal, therefore the problem of low transformation was not with the cells.

To check the ligation reaction was not the cause of the low transformation an experiment was performed using uncut M13, cut M13 and re-ligated M13 (pUC was also tested in the same manner). When these were transformed into E.coli, the uncut M13 or pUC and the re-ligated vector all transformed successfully whilst the cut vector did not yield any transformants as would be expected. Consequently the



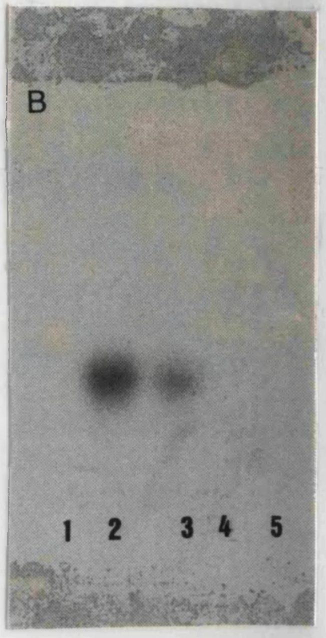


Figure 31. PCR DNA and Southern Blot.

Figure A. Shows a 1% agarose gel showing the DNA obtained by the polymerase chain reaction. Lane 1. Lambda Pst cut DNA; Lane 2. PCR products showing a larger primer dimer band and a smaller single product band produced using 10mM $MgCl_2$ Lane 3. As lane 2 only 20mM $MgCl_2$ was used. Lane 3. Primer dimer only formed when 30mM $MgCl_2$ was used. **Figure B**. Shows the autoradiograph of the gel. A positive hybridisation can be seen by the presence of a single band which corresponds to the PCR DNA in lanes 2 & 3 of figure A. This shows that the PCR DNA contains part of the calmodulin gene.

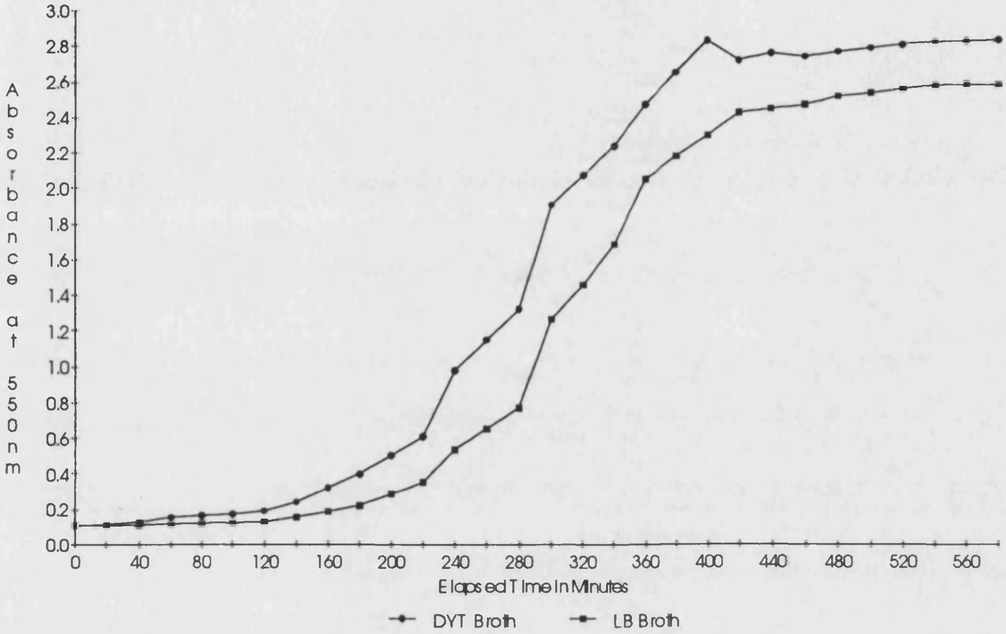


Figure 32. Growth Curve for <u>Escherichi coli</u>.

A loopful of <u>E.coli</u> were placed in either double yeast tryptone broth or Luria bertini broth and incubated at 37°C. At 20 minute intervals 1ml of the broth was removed and its absorbance measured at 550nm.

ligation reaction was also not the source of the low transformation rate. This experiment also showed that the problem was not due to the competent cells being 'incompetent'.

As there appeared to be no problem with either the vectors, the cells or the ligation enzymes there was only one other possible explanation. The fragment of DNA that was being inserted into the vector was PCR generated DNA. Consequently it is not phosphorylated and this can cause problems with ligation into vectors composed of phosphorylated DNA. Whilst it is possible to phosphorylate the PCR DNA a large proportion of the PCR DNA is lost in the reaction involved so that this is not a favoured option. An experiment was designed to asses the effects of using a phosphorylated and de-phosphorylated vector in the ligation reaction. pUC or M13 were either cut in its native phosphorylated form or it was treated with phosphatase before being cut. The cut vector was then ligated and transformed into E.coli. Treating the vector with phosphatase decreases the chances of it re-ligating to itself and therefore increases the likelihood of the PCR DNA inserting itself into the vector. It was found that phosphatased treated pUC resulted in more transformants than the phosphorylated pUC. A third type of pUC vector was tried which had been treated with SMA-1 BAP, this yielded even higher levels of transformants and so this was used as a vector for ligation with the PCR product.

Furthermore during these experiments the range of temperature used in the ligation reaction was varied, so that ligations were performed at 14°C, 15°C and room temperature. It was found that when using the SMA-1 BAP vector that room temperature gave the highest number of transformants after ligation. When phosphorylated or de-phosphorylated pUC was used, the temperature in the ligation reaction appeared to have little effect on the rate of transformation.

At this stage it was decided to try cutting the pUC with HincII and EcoRI to produce both blunt ended and sticky ended vectors. Even a vector cut with both, giving rise to a blunt and sticky end on one vector were tried. Possible positive plaques were found as a result of HincII cut pUC and PCR DNA. PCR DNA was ligated into HincII cut pUC and transformed into E.coli. Initially, none of the cells transformed, this was probably due to the presence of some contaminating poison. The ligation and transformation reactions were repeated several times using both phosphorylated and de-phosphorylated vector and PCR product. However, the cells would still not transform. In later transformations it was found that uncut vector would transform whilst cut and re-ligated vector or vector ligated with PCR product would not transform at all, suggesting that the ligation reaction was not working.

3.1.5 Mini-Preparations of Positive Transformants

Isolated DNA from the mini-preparation of positive transformants together with the DNA of a control transformant was analysed by 1% agarose gel electrophoresis. In many cases there was no size difference so no further processing was performed. However if the DNA from the positive transformant was of a higher molecular weight than that from the transformant not containing any inserted vector, then the PCR DNA had successfully inserted into the vector. In one case there was a size difference and a restriction digest was performed using EcoR1. Unfortunately the DNA was resistant to digestion by EcoR1, as when analysed on a 1% gel the 'digested' DNA migrated the same distance as undigested DNA from the positive transformant. Consequently this meant that it couldn't be processed further.

During the course of the transformation experiments it became necessary to amplify the PCR DNA. To amplify the remaining PCR product, a new 31 base 3' oligonucleotide primer was synthesised to use with the existing 42 base 5' oligonucleotide primer (section 2.2.5.1). This was a shortened version of the existing 3' primer:

3'-GAG-CAC-AAG-CTT-CAC-CTC-CTC-GTC-GGT-TAA-C.

incorporating the four restriction sites, HpaI, HgiAI, HindIII, and AluI. This primer was then used in PCR reaction using the previously obtained PCR DNA as the template. However, on 1% agarose gel analysis only primer dimer was found. On successive attempts PCR product was produced however the DNA was less than 1.5kb and did not form a tight band on agarose gel electrophoresis. Consequently it was not usable.

3.1.6 RNA Isolation

Typical values for the isolated RNA are shown in table 7. In general all three types of RNA were successfully isolated, mRNA, tRNA and rRNA. See figure 33. The purity ratio tended to vary from one preparation to another. But, in general the RNA was of good quality and so usable for further processing.

3.1.7 mRNA Isolation

Initial isolations of mRNA produced RNA with a concentration of 0.116mg/ml, and a purity ratio of 2.78. However when a drop of the final 'mRNA' was mixed with a drop of ethidium bromide no fluorescence was visible. It was thought that the mRNA may not have been isolated successfully due to the presence of sodium dodecyl sulphate in the original RNA samples. Consequently in subsequent isolations of

Concentration of RNA (mg/ml)	Purity Ratio	Analysis on gel
0.44	-	rRNA, tRNA and mRNA present.
14.79	1.58	showed signs of degradation and only one band present.
4.23	1.61	All three bands of RNA present.
2.08	1.84	All three bands of RNA present.

Table 7. Typical Quantities of RNA Isolated.

The table shows the mean data obtained from four different batches of tapeworm tissue that were used for isolating RNA. In nearly all extraction procedures all three bands of RNA were preserved, as determined by denaturing agarose gel electrophoresis. The purity ratio is calculated by dividing the absorbance at 260nm by the absorance at 280nm.

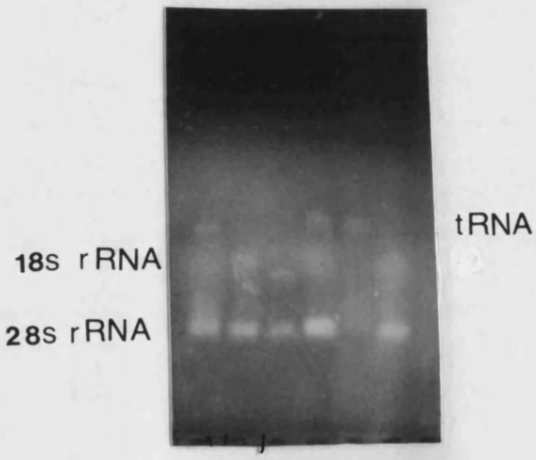


Figure 33. 1% Formaldehyde Agarose gel of RNA Samples.

Isolated RNA was analysed by denaturing agarose gel electrophoresis. Three bands are visible in each sample which correspond to tRNA, mRNA and rRNA.

mRNA the sodium dodecyl sulphate was removed from the RNA sample before application on the oligo dT column.

Subsequent isolations of mRNA had concentrations of 0.16mg/ml and a purity ratio of 1.82. When analysed on a denaturing agarose gel a smear of RNA was present from the base of the gel to the middle. Consequently these samples were used for first strand synthesis of the mRNA as described in section 2.2.11.

Once cDNA synthesis was completed a sample of the radiolabelled cDNA was analysed by alkaline gel electrophoresis. Ideally the first strand cDNA should appear as a smear ranging in size from 500 bases to 8kb. However, there was no evidence of synthesis having occurred. There are two possible explanations for this, one is that there may have been secondary structures present on the mRNA, which impeded the progress of the reverse transcriptase. The second reason is that the mRNA could have been degraded by RNAases before incubation.

3.2 Protein Biochemistry

3.2.1 Development of an Isolation Procedure for Calmodulin

Initially only a two step method was used which involved a heat treatment step followed by phenyl Sepharose hydrophobic chromatography with no further processing by ion exchange chromatography (section 2.3.1.1).

After heat treatment and centrifugation of the homogenate the resulting clarified supernatant was applied to a phenyl Sepharose column. The column was sequentially washed before calmodulin was eluted using a buffer containing EDTA/EGTA. A typical elution profile obtained on addition of the EDTA/EGTA buffer is shown in figure 34. It can be seen that there are three peaks, instead of one as expected. The fractions forming each of these peaks were pooled and together with samples collected throughout the isolation procedure, the protein content determined. The percentage protein recovered can be seen in table 8.

After heat treatment, 50% of the protein is removed, with a further 47% being removed upon centrifugation of the heat treated supernatant. Most of the remaining 3% of the protein does not interact with the phenyl Sepharose column so that only 20% of the applied protein adsorbs to the matrix. The quantity of calmodulin recovered from the column represented 0.8% of the total protein in the original homogenate.

On analysis of the fractions forming the three peaks, by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) it was found that fractions from the first peak contained a mixture of proteins including calmodulin. See figure 35A.

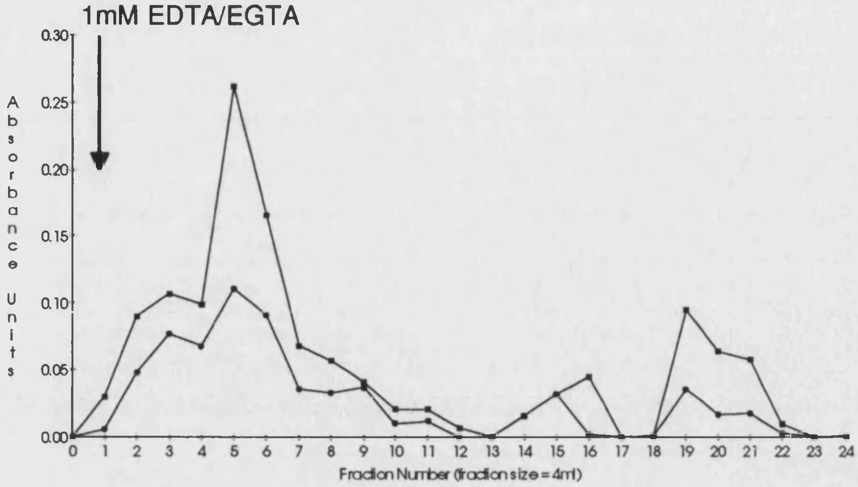


Figure 34. Elution Profile for Phenyl Sepharose Chromatography.

Pig thymus homogenate was heat treated as per method 1 (section 2.3.1.2.) and the resulting supernatant applied to a phenyl Sepharose column, which was run at a flow rate of 2ml per minute. The elution profile depicted, shows the protein peaks which formed after application of buffer containing 1mM EDTA.

SAMPLE		CONCENTRATION OF SAMPLE (mg/ml)	PERCENTAGE PROTEIN OF HOMOGENATE PROTEIN (%)
Homogenisation and heat treatment of homogenate	Initial Homogenate	23	100
	Heat Treated Supernatant	11.5	50
	Supernatant of centrifuged heat treated supernatant	0.63	2.7
Phenyl Sepharose Fractions	Sample application Eluent	0.52	2.2
	Wash with Tris buffer containing 0.1mM $CaCl_2$	0.27	1.13
	Wash with Tris buffer containing 0.1mM $CaCl_2$ and 0.4M NaCl.	0.26	1.11
	Calmodulin fraction (elution with Tris buffer containing 1mM EDTA).	0.17	0.76

Table 8. Protein recovery from Pig thymus

This shows the amount of protein isolated as a percentage of the protein in the homogenate recovered from pig thymus using method 1 to isolate the calmodulin.

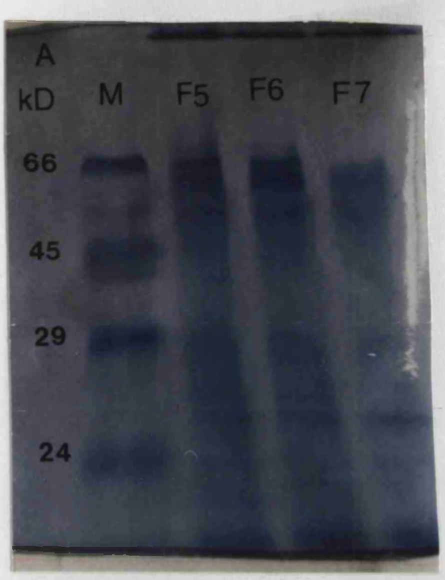


Figure A.

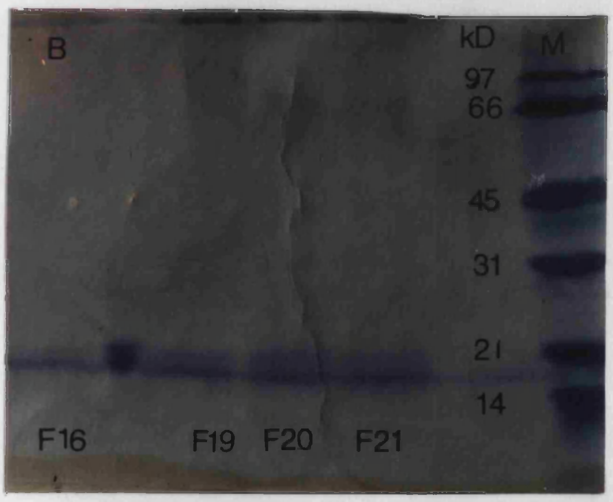


Figure B.

Figures 35A & B. SDS-PAGE of Phenyl Sepharose Fractions.

The fractions forming the peaks in figure 34 were pooled and concentrated prior to analysis using 20% SDS-PAGE. The proteins found in the first peak are shown in figure A, whilst those in the second and third peak are shown in figure B. M=molecular weight markers and kD=kilo Daltons. F5, F6 and F7 refer to fractions' 5,6, and 7 from figure 34; F16, F19, F20 and F21 similarly refer to fractions 16, 19, 20 and 21.

The second two peaks from the column were found to contain one or two contaminating proteins and calmodulin, which migrated with a molecular weight between 14kD and 21kD. See figure 35B. It can be seen from the results of the gel electrophoresis that the calmodulin has come off throughout the application of the elution buffer, rather than as a discrete peak. This could indicate that the flow rate at which the column was run was too fast. Consequently, the extraction was repeated but decreasing the flow rate to 0.5ml per minute instead of 2ml per minute, which was the flow rate achieved under gravity and used in these extractions.

When the phenyl Sepharose column was run at a flow rate of 0.5ml per minute, only one peak formed on application of the EDTA/EGTA buffer. The elution profile is shown in figure 36. However, on this occasion the calmodulin fraction only contained 0.3% of the total protein found in the homogenate. See table 9. It is also evident that the heat treated supernatant contained only 20% of the total protein in the homogenate. This can only be explained by differences in the batches of pig thymus used. However, it does mean that there is less protein to interact with the gel matrix, so that the lower yield of calmodulin is not surprising.

The calmodulin fractions were analysed by SDS-PAGE. See figure 37. This revealed that they contained eight other proteins with molecular weights of 200kD, two of ≈50kD, 45kD, 40kD, 36kD, 29kD, and less than 14kD. However, the band representing calmodulin is much more densely stained than any of the other bands. These other proteins may either be calcium binding proteins in their own right, and hence interact with the phenyl sepharose in the same manner as calmodulin, i.e. upon binding calcium they undergo a calcium induced conformational change exposing residues, which enables them to interact with the phenyl Sepharose.

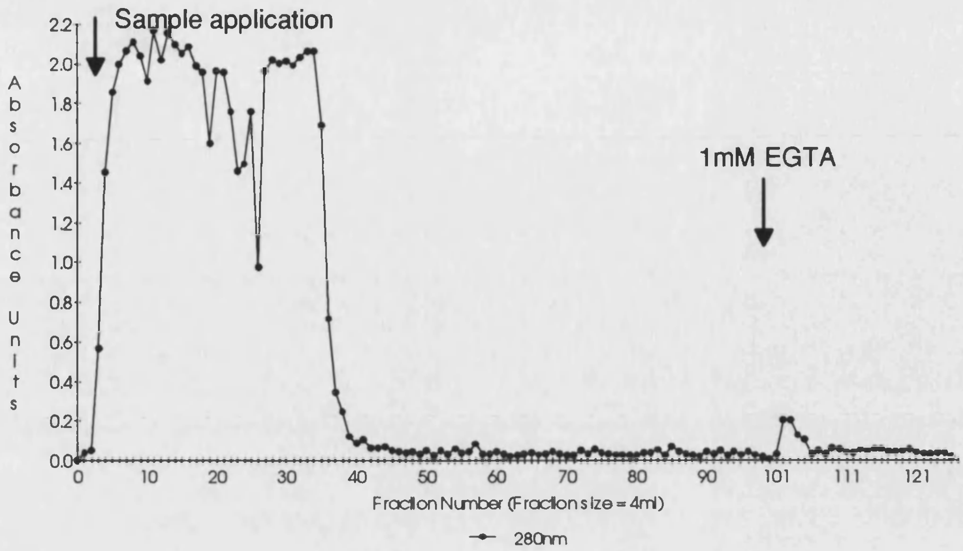


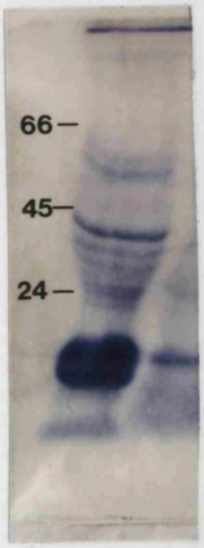
Figure 36. Elution Profile of Phenyl Sepharose

Pig thymus homogenate was heat treated as per method 1 (section 2.3.1.1.) and applied to a phenyl Sepharose column at a flow rate of 500µl per minute. Only one peak of protein was eluted on application of the buffer containing 1mM EGTA.

Sample	Total Protein Content (g)	Percentage Protein of initial homogenate (%)
Initial homogenate	2.5	100
Initial supernatant	0.9	36
Initial pellet	1.6	64
Heat Treated Supernatant	0.51	20
Phenyl Sepharose Calmodulin Peak	0.7mg	0.3

Table 9. Protein Recovery for Pig Thymus

This shows the quantity of protein recovered using method 1 to isolate calmodulin from pig thymus as a percentage of the protein in the homogenate. In this extraction scheme the flow rate for the phenyl Sepharose chromatography was reduced from 2ml/minute to 0.5ml/minute.



Calmodulin

Figure 37. SDS-PAGE of the Calmodulin Peak

Fractions forming the peak (figure 36) on application of buffer containing EGTA were pooled and concentrated prior to gel analysis. A very dark staining band can be seen between 14 and 20kD representing calmodulin. However, there are several contaminating proteins.

Then on application of the elution buffer containing EDTA or EGTA the binding is reversed so freeing the proteins into solution. The other possible identity of these proteins is that they may in fact be proteins to which calmodulin has interacted or forms a permanent domain. The former calmodulin/calcium protein complexes will dissociate upon the addition of the elution buffer, so freeing the calmodulin and the protein into solution. Due to the presence of the contaminating proteins in the calmodulin fractions, an alternative approach was used where the heat treatment step was replaced by an ammonium sulphate precipitation and an isoelectric precipitation step (section 2.3.1.2.).

The supernatant resulting from the centrifuged pig thymus homogenate was first brought to 50% (w/v) ammonium sulphate before being centrifuged. The resulting supernatant was then iso-electrically precipitated by adjusting the pH to 4.0. After centrifugation the pellet containing calmodulin was resuspended and applied to a phenyl Sepharose column, which was run at the slower flow rate of 0.5ml/minute. However, this proved rather disappointing, as no peak was detectable by absorbance at 280nm, after the addition of the calmodulin eluting buffer. The fractions obtained with each solution applied to the column were collected and pooled, and the protein content determined. See table 10.

From this it can be seen that the calmodulin fractions contain 13% of the original protein in the homogenate. This is a much higher yield of protein in the calmodulin fraction than was obtained using the heat treatment method. As protein had been found in these fractions they were analysed on a polyacrylamide gel. See Figure 38. The gel shows fractions from different points within the isolation procedure so one can see the effects of the different treatments. Calmodulin can be seen as quite a strong band in the lane showing the resuspended pellet obtained from the isoelectric precipitation step. However, the final calmodulin containing fraction still contains other unwanted proteins, which is to be expected considering the relatively high protein content. It was thought that these proteins could be removed by applying the pooled calmodulin fractions to a DEAE-ion exchange column (as described in section 2.3.1.1).

An imidazole buffer, pH6.1 was chosen for the DEAE ion exchange chromatography for two reasons. The first is that calmodulin from rat testis has been shown to elute consistently between 0.2 and 0.3M NaCl when an imidazole buffer was used at pH's ranging from 7.8 to 5.5. It is thought that this is due to calmodulins' single histidine and its highly acidic nature. The second reason is that imidazole is a cationic buffer, with a pKa 6.9., which means that unlike anionic buffers, it is less likely to interact with the positively charged matrix of the DEAE-cellulose. Consequently the pH of the buffer within the column should remain constant throughout the chromatography (Dedman & Kaetzel 1983).

The pooled fractions containing the calmodulin were therefore applied to a DEAE52 cellulose ion exchange column. On application of the high salt buffer (0.4M NaCl) calmodulin eluted as a single peak and was found to have no other contaminating protein present when analysed by SDS-PAGE. See figure 39A and B.

Sample	Protein Content (mg/ml)	Percentage Protein of homogenate (%)
Initial Homogenate	9.75	100
Initial Supernatant	4.75	48
Ammonium Sulphate Precipitation Supernatant	2.85	29
Isoelectric Precipitation With Ammonium Sulphate Discarded Supernatant	600µg/ml	6
Resuspended Isoelectric Precipitation Pellet	1.75	18
Phenyl Sepharose Calmodulin Fraction	1.3	13

Table 10. Protein Recovery of Method 2 Isolation Procedure for Calmodulin for Pig Thymus

Pig thymus was homogenized and subjected to ammonium sulphate precipitation followed by isoelectric precipitation prior to application to a phenyl Sepharose column.

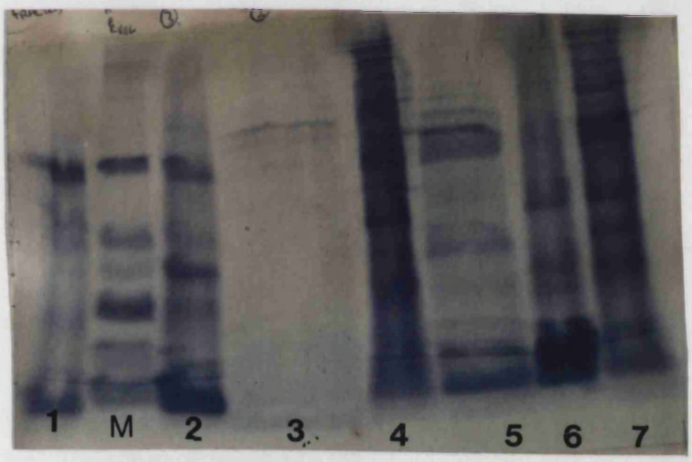


Figure 38. SDS-PAGE of Samples from Method 2

Samples were analysed on a 20% polyacrylamide gel. M= molecular weight markers: 66, 45, 36, 29, 24 and 20kD, lane 1= phenyl Sepharose calmodulin fraction, lane 2 and 3=$NH_4(SO_4)_2$ isoelectric precipitation pellet and supernatant, lane 4 and 5= $NH_4(SO_4)_2$ precipitation pellet and supernatant, lane 6=initial pellet and lane 7=initial supernatant.

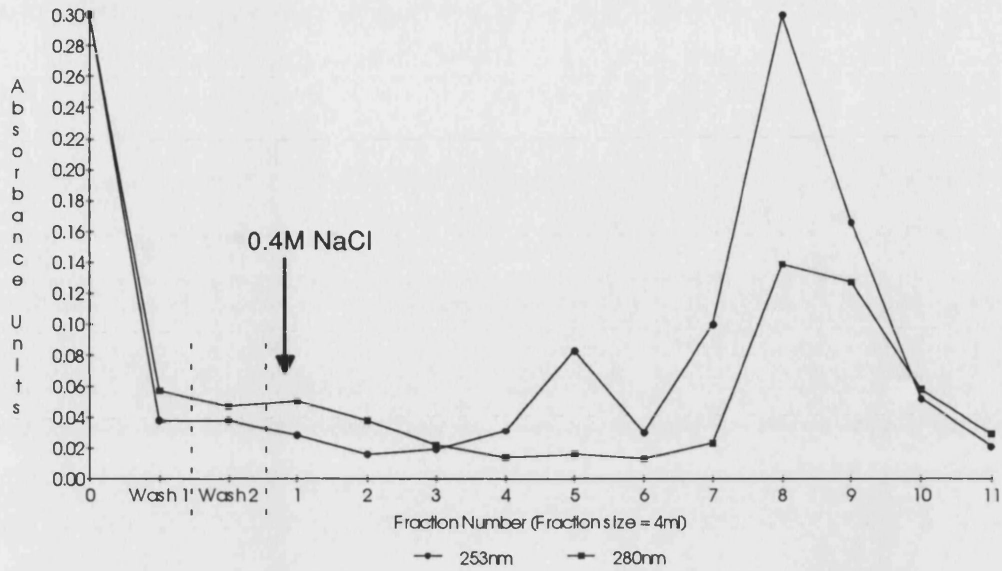


Figure 39A. Elution Profile for DEAE Chromatography

The resulting phenyl Sepharose calmodulin fraction, after ammonium sulphate precipitation, shown in figure 38, was applied to a DEAE-ion exchange column to remove contaminating proteins. Calmodulin appears to elute in one major peak.

Figure 39 B

Figure 39 C

Figure 39B & C. SDS-PAGE of DEAE Calmodulin Fractions

The pig thymus calmodulin fraction is clear of any contaminating protein (B). When run in the presence and absence of calcium it shows a migratory shift running at around 20kD in the presence of EGTA, and 16kD with calcium (C).

To verify that this protein band was calmodulin, a sample was run on an SDS-polyacrylamide gel in the presence and absence of calcium. The result can be seen in figure 39C. Calmodulin and the whole family of EF-Hand calcium binding proteins, e.g. muscle troponin C, have different electrophoretic mobility's in the presence and absence of calcium. On SDS-PAGE calmodulin migrates at between 19kD and 24kD in the presence of EDTA or EGTA, whereas in the presence of calcium calmodulin migrates further, to around 16kD. The reason for this anomalous behaviour is not fully understood but it is thought to relate to the stabilising effect of calcium on the calmodulin molecule. When calcium is bound to calmodulin, it is more resistant to the denaturing effects of sodium dodecyl sulphate, so that it does not unfold to the same extent as when it is in the apocalcium form, thus giving rise to the different electrophoretic behaviour (Burgess, Jemiolo & Kretsinger 1980; Watterson et al. 1976; Klee, Crouch & Krinks 1979; Grab et al. 1979; Klee & Vanaman 1982).

At this time an alternative method, which is used to isolate calmodulin for nuclear magnetic resonance studies, was suggested by H.Vogel (1991). The method involved two precipitation steps, firstly a precipitation with trichloroacetic acid followed by an ammonium sulphate precipitation as already used. (section 2.3.1.3 and 2.3.1.1). After the isoelectric precipitation with ammonium sulphate, the resulting supernatant was applied to a DEAE cellulose ion exchange column. It was felt that if this was performed prior to the phenyl sepharose chromatography more unwanted proteins would be removed. The overall extraction scheme is shown in figure 40.

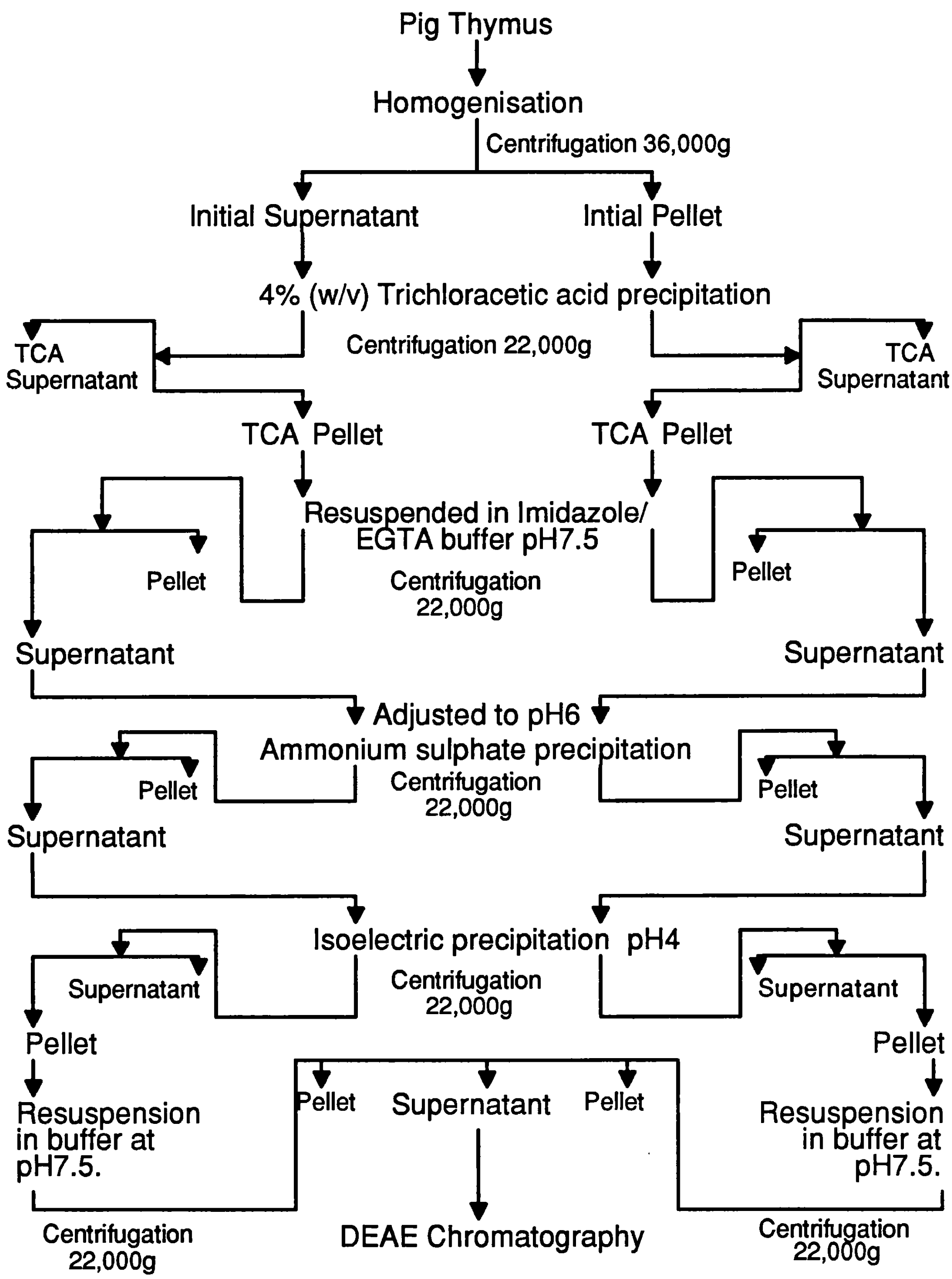


Figure 40. Flow Diagram of TCA Extraction Procedure

Tissue is homogenized and centrifuged prior to Trichloroacetic acid (TCA) precipitation. Ammonium sulphate precipitation followed TCA precipitation with the resulting pellet being resuspended, brought to pH7.5 and centrifuged. The final supernatant was then applied to a DEAE ion exchange column.

The elution profile of the DEAE ion exchange column is shown in figure 41. Two columns were run, one for the supernatant resulting from treatment of the initial supernatant, and another for the supernatant resulting from processing of the initial pellet. In both cases a single peak forms on application of imidazole buffer, containing 0.4M NaCl.

The protein recovery obtained from the different stages of this isolation method are shown in table 11. It would appear that the protein content of the initial pellet has been enriched, so that it contains 126% of the protein content of the homogenate whilst the initial supernatant contains 50% of the homogenate. After the TCA precipitation more protein is recovered from the initial supernatant sample than the initial pellet. This follows for the various fractions obtained from the DEAE column and the phenyl Sepharose with the resulting calmodulin containing fractions from the initial supernatant representing 2.2% of the original homogenate. This recovery is also much higher than that obtained using either method one or two. Very little protein could be detected in the calmodulin samples from the initial pellet.

Before applying the calmodulin samples to a phenyl Sepharose column samples of the different stages in the extraction procedure were analysed by SDS-PAGE, the results of which can be seen in figure 42A and B. It will be noted that the calmodulin containing fractions still contain other contaminating proteins consequently these fractions were pooled and applied to a phenyl Sepharose column. On application of the EDTA/EGTA buffer to the phenyl Sepharose column, three potential calmodulin peaks were found however when these were analysed by SDS-PAGE no visible band of protein was present.

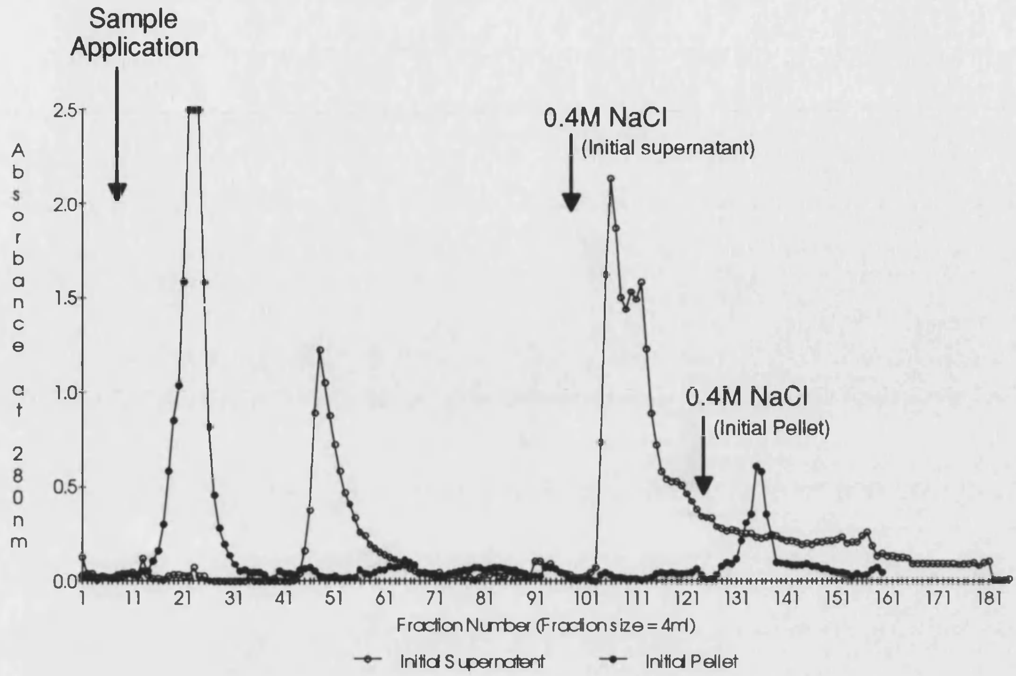


Figure 41. Elution Profile for DEAE ion Exchange Chromatography Using Method 3 to Isolate Calmodulin

Pig thymus homogenate was processed according to method 3, which is shown schematically in figure 40. The final supernatant resulting from the processing of both the initial supernatant and pellet were applied to separate DEAE-cellulose columns. The calmodulin containing fractions were eluted by 0.4M NaCl in imidazole buffer. As can be seen a single peak forms on each occasion.

Sample		Protein Content (mg/ml)	Percentage Protein of homogenate (%)
Homogenate		59	100
Initial Supernatant		29.5	50
Initial Pellet		74.5mg/g	126
Trichloroacetic acid supernatant 1. (Discarded)	From initial supernatant	0.3	0.5
	From initial pellet	0.9	1.5
Trichloroacetic acid supernatant 2 (retained)	From initial supernatant	9	15
	From initial pellet	2.75	4
Ammonium sulphate end resuspended pellet	From initial supernatant	7.5	12
	From initial pellet	nd	-
DEAE52 Chromatography 0.15M NaCl wash.	From initial supernatant	8.75	14
	From initial pellet	0.58	0.9
DEAE52 Chromatography 0.4M NaCl wash (Calmodulin containing)	From initial supernatant	1.35	2.2
	From initial pellet	0.01	0.01
Phenyl sepharose Calmodulin fraction	From initial supernatant	1.63	2.76
	From initial pellet	nd	-

Table 11. Shows the Protein Recovery from Method 3

Pig thymus was treated as per method 3 (section 2.3.1.3.), shown schematically in figure 40. Samples were taken and analysed throughout. [nd = none-detected].

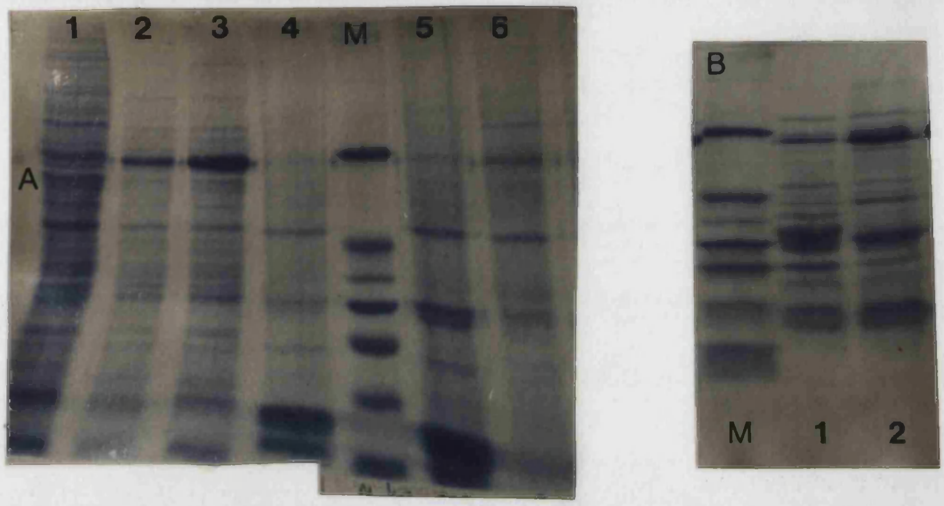


Figure 42. SDS-PAGE of Samples Obtained Throughout Method 3

Figure A. Lanes 1,2 and 3= homogenate, initial supernatant and pellet. Lane 4= pellet from TCA precipitated initial pellet. Lane 5 and 6=supernatant resulting form centrifugation of resuspended TCA pellets of initial supernatant and pellet. **Figure B.** Lane 1= fractions 111-121 from DEAE column of initial supernatant. Lane 2= fractions 105-110 of DEAE column of initial supernatant. M= molecular weight markers of 66, 45, 36, 29, 24, 20 and 14kD.

Whilst the TCA procedure gave reasonable results, it was felt that it involved too many steps and was a rather lengthy procedure whereas the combination of ammonium sulphate precipitation followed by phenyl Sepharose and ion exchange chromatography yielded a relatively pure fraction of calmodulin. Another factor in deciding against the TCA method, and also the heat treatment method, was that both of these treatments can result in deamidation of asparaginyl and glutaminyl residues resulting in minor modifications of the calmodulin molecule. The consequence of which can be a biologically inactive molecule that would be relatively useless for any further investigations (Haiech et al. 1981; Klee & Vanaman 1982).

As already stated, it was also felt that if the ion-exchange step was performed after the ammonium sulphate precipitation step and before the phenyl Sepharose chromatography, more unwanted protein would be removed. This would then lead to less competition for binding sites on the phenyl Sepharose and enable more calmodulin to bind to the hydrophobic gel matrix so yielding a purer preparation of calmodulin. The scheme for the final method tried, which took into account these various factors, is shown in figure 43 (detailed in section 2.3.1.4.).

This method was tried repeatedly with pig thymus, and a typical elution profile for both the DEAE and the phenyl Sepharose chromatography are shown in figure 44. The DEAE chromatography yielded a single sharp peak on application of the high salt buffer. Similarly, a single peak was also observed on application of the EDTA/EGTA buffer to the phenyl Sepharose column.

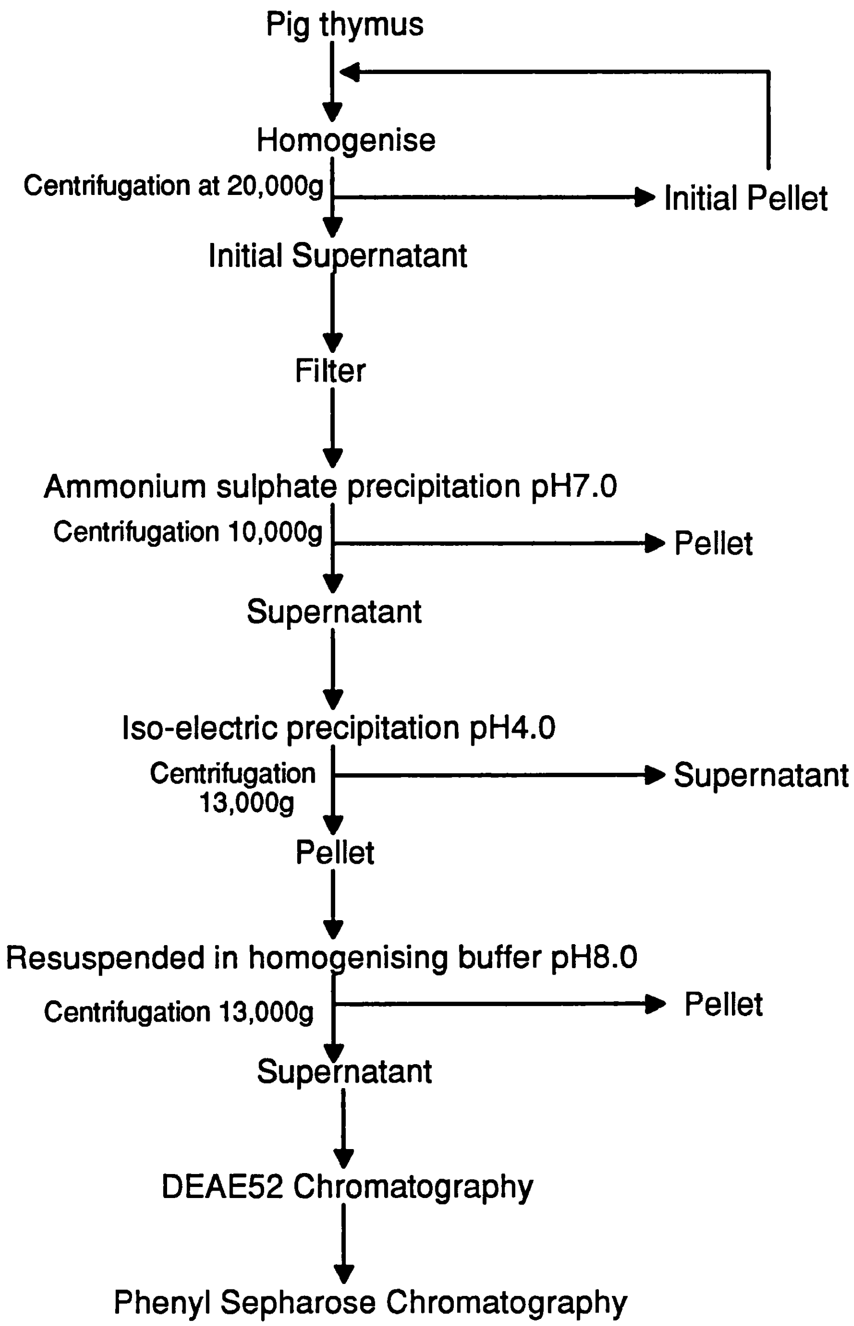


Figure 43. Schematic Diagram of the final Calmodulin Isolation Procedure

This method was the method that was finally used to isolate calmodulin from both pig thymus and tapeworm. The method is quick and can be completed in 2 to 3 days. Homogenate protein is precipitated with ammonium sulphate prior to application to an ion-exchange column and finally phenyl Sepharose chromatography.

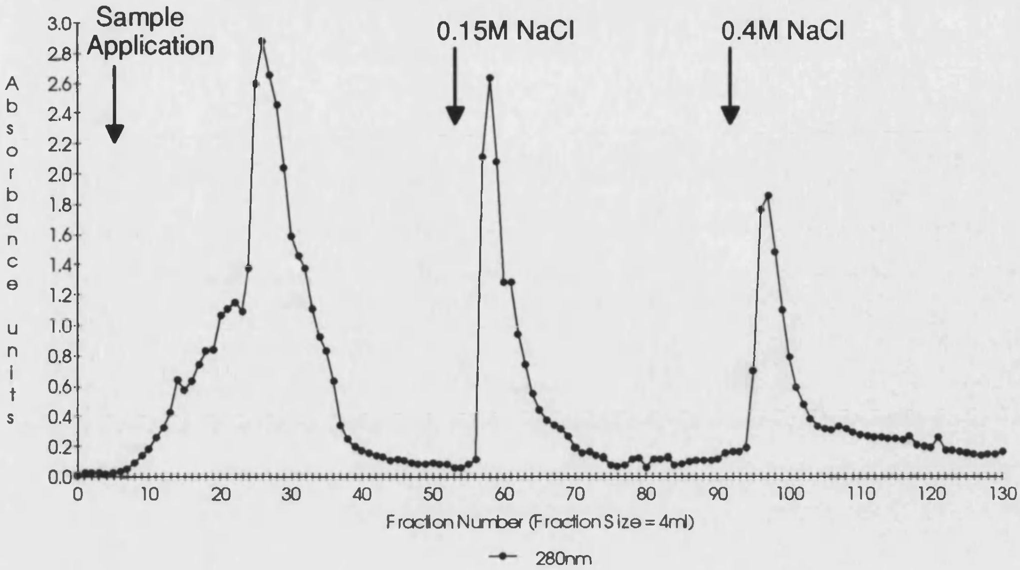


Figure 44A. Method 4: Elution Profile for DEAE52 Chromatography

Pig thymus homogenate was treated with ammonium sulphate, and centrifuged prior to application to a DEAE52 cellulose column. Well defined peaks form on application of each of the elution buffers.

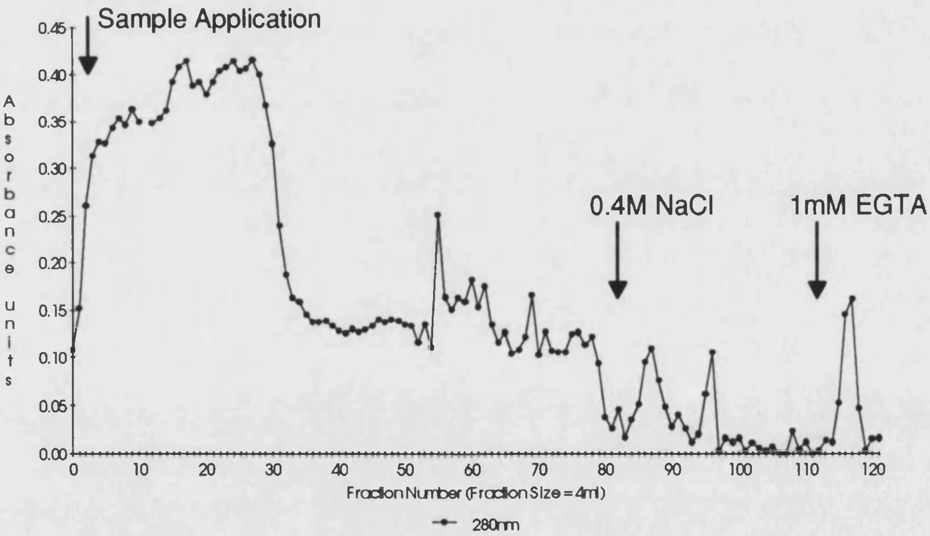


Figure 44B. Method 4: Elution Profile for Phenyl Sepharose

The final peak obtained from the DEAE chromatography, shown in figure 44A, was applied to a phenyl Sepharose column. A small, clean, calmodulin peak was observed.

The overall protein recovery is shown in table 12. It is evident that 85% of the protein in pig thymus is insoluble, so being removed by the first centrifugation step. However, the total protein found in both the ammonium sulphate precipitation samples is greater than that recorded for the initial supernatant. The ammonium sulphate supernatant contained 1.32g of protein whilst the pellet contained 1.58g; a combined total of 2.9g, which represents 42% of the protein in the homogenate. This figure is more in line with that recorded for previous isolations. Consequently, the initial supernatant figure must be an under estimate. The isoelectric precipitation of the ammonium sulphate supernatant removed 0.391g of protein, which is equivalent to 29% of the protein present in the ammonium sulphate supernatant. A further 24% (0.31g) was removed by DEAE chromatography. After phenyl Sepharose chromatography the final calmodulin fraction contained 4.3mg protein, which represented 0.06% of the total protein.

This method also appeared to produce a relatively uncontaminated sample of calmodulin as can be seen in figure 45. Therefore this method was selected as the favoured method for trying to isolate calmodulin from H.diminuta.

Tapeworm tissue was homogenised, precipitated with ammonium sulphate and applied to a DEAE column, the elution profile for which can be seen in figure 46A. On addition of the 0.4M NaCl, two peaks formed instead of the one seen with pig thymus. Both of these peaks were pooled and applied to a phenyl Sepharose column. The elution profile for this is shown in figure 46B. A small calmodulin peak is observed.

Sample	Total Protein Content (mg)	Percentage protein of homogenate (%)
Initial Homogenate	6.79g	100
Initial Supernatant	958	14
Initial Pellet	5.84	86
Ammonium sulphate supernatant	1.32g	19
Ammonium sulphate pellet (discarded)	1.58g	23
Ammonium sulphate isoelectric precipitation supernatant (discarded)	391	5.8
DEAE Application Eluent	240	3.5
DEAE 0.15M NaCl Wash	71.5	1.1
Phenyl sepharose application eluent	42.2	0.62
Phenyl sepharose 0.4M NaCl wash	2.07	0.03
Phenyl sepharose calmodulin fraction	4.25	0.06

Table 12. Protein Recovery From Method 4

The protein content of samples from each stage of the isolation procedure was determined by Coomassie blue assay. The protein content is shown as a percentage of the protein content of the homogenate.

Figure 45. SDS-PAGE of the Calmodulin Obtained Using Method 4

On analysis of the pig thymus calmodulin fraction, resulting from the phenyl Sepharose chromatography, it appears to be free of contaminating proteins.

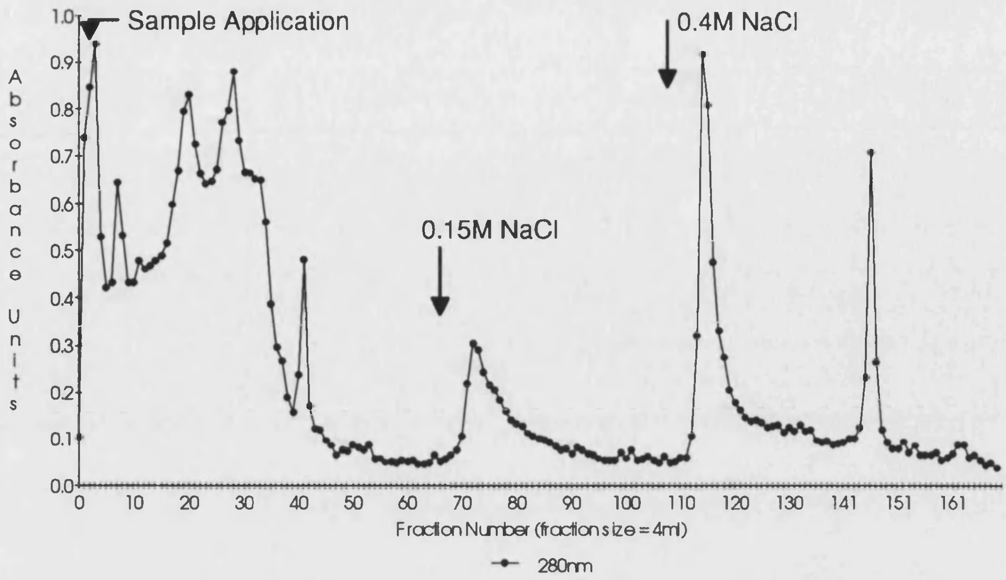


Figure 46A. DEAE Elution Profile for Tapeworm Calmodulin

Tapeworm tissue was treated as per method 4 and applied to a DEAE column. Two peaks formed on application of the high salt wash.

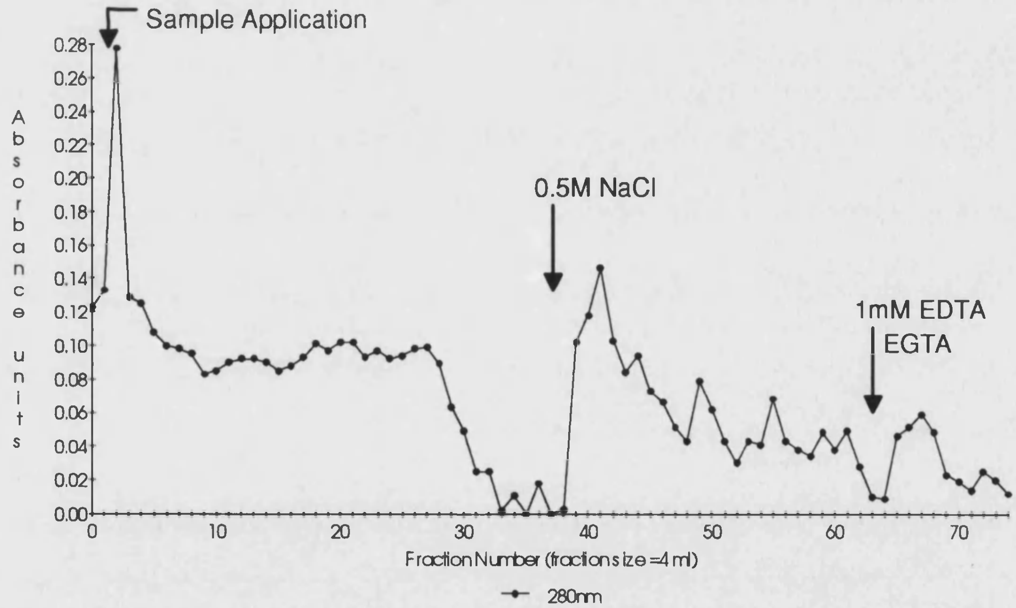


Figure 46B. Phenyl Sepharose Elution Profile for Tapeworm Calmodulin

The two peaks from the 0.4M NaCl wash in figure 46A were applied to a phenyl Sepharose column. A small calmodulin peak formed on application of EDTA/EGTA.

Analysis of the protein content of the samples taken throughout the isolation procedure showed that the calmodulin fraction contained 0.41% of the total homogenate, which is higher than that recorded for pig thymus. See table 13.

Another noticeable difference between the tapeworm protein distribution and that found in pig thymus, is that a much larger percentage appears to be soluble in tapeworm because 98% of the total protein appeared in the initial supernatant, compared to 50% observed for pig thymus. Ammonium sulphate precipitation of the initial supernatant removes 57% of the protein, with a further 9% being lost in the isoelectric precipitation. After DEAE chromatography there is only 1.8% of the total protein remaining, and as already stated after phenyl sepharose chromatography the calmodulin fraction contains 3.65mg protein equivalent to 0.41% of the total protein in the homogenate.

When the samples were analysed by SDS-PAGE the calmodulin fraction showed contamination with other proteins. There was also evidence of proteolytic degradation of the proteins throughout the procedure, by the presence of a continuous smear of Coomassie dye from around 20kD and below in some of the fractions. Consequently in all subsequent isolations PMSF and mercaptoethanol were included in all the buffers to minimise the effects of proteolytic enzymes.

In an attempt to remove the unwanted proteins from the tapeworm calmodulin fraction the sample was applied to a G50 Sephadex size exclusion matrix. Two peaks were eluted, one containing protein greater than 30kD and the other containing proteins smaller than 30kD including calmodulin. See figure 47.

Sample	Protein Concentration (mg/ml)	Total Protein (mg)	Protein as a percentage of homogenate (%)
Homogenate		887.42	100
Initial supernatant	3.43	874.7	98.6
Initial Pellet	6.36 mg/g pellet	12.72	1.4
Ammonium Sulphate Precipitation Pellet (discarded)	33mg/g pellet	504.9	56.9
Ammonium sulphate isoelectric precipitation supernatant (discarded)	0.63	81.9	9.23
Ammonium sulphate isoelectric precipitation pellet (retained)	30mg/g pellet	70.2	7.9
DEAE Chromatography (see figure 46A for elution profile)			
Application eluent fractions 1-28	0.46	51.5	5.8
Application wash fractions 29-69	0.18	28.8	3.2
0.15M NaCl wash fractions 70-89	0.13	9.88	1.1
0.15M NaCl wash fractions 90-113	0.04	3.68	0.4
0.4M NaCl wash fractions 114-150	0.15	15.6	1.75
Phenyl sepharose chromatography (see figure 46B for elution profile)			
Application eluent fractions 1-39	0.09	13.73	1.55
0.5M NaCl Wash fractions 40-65	0.08	3.75	0.42
EGTA/EDTA wash fractions 66-75 (Calmodulin)	0.08	3.65	0.41

Table 13. Protein Recovery for Tapeworm Using Method 4

This table shows the protein recovered from each of the steps used in isolating tapeworm calmodulin using method 4. The most stiking difference between pig thymus and tapeworm is the large percentage of soluble protein present in the worm, reflected in the high percentage of protein found in the initial supernatant.

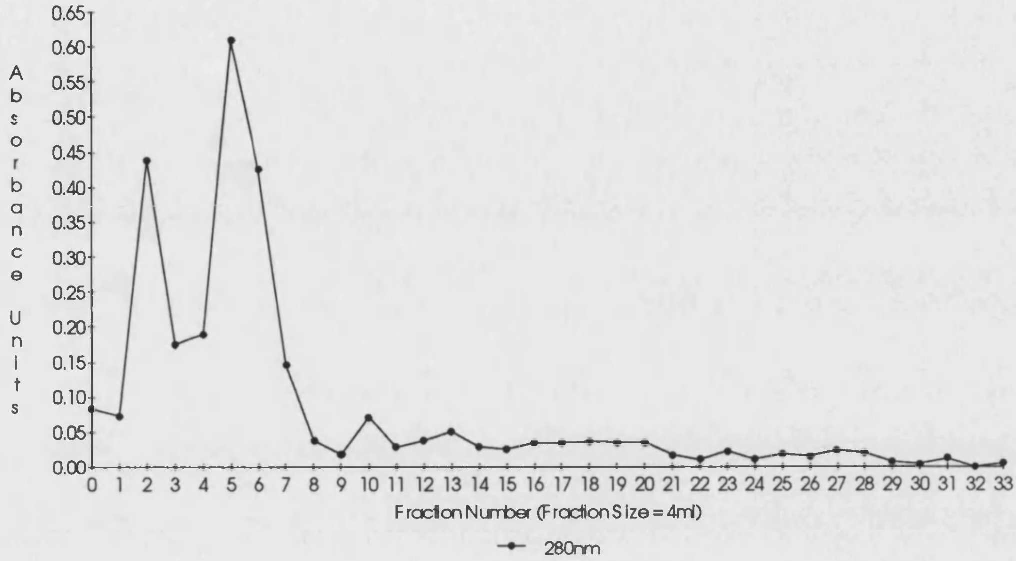


Figure 47. G50 Sephadex Size Exclusion Chromatography of Tapeworm Calmodulin

The phenyl Sepharose fractions containing calmodulin were applied to a G50 Sephadex column. The void volume was found to be 14 ml using blue dextran consequently fractions 1-4 are proteins which are greater than 30kD, and cannot enter the gel matrix. Fractions 5-8 are proteins that can enter the gel bed and are smaller than 30kD.

To further quantify the effectiveness of the method 4 isolation procedure, it was used to isolate calmodulin from rat testis, which is known to be the second richest source of calmodulin, after bovine brain. The protein recovery for which is shown in table 14. Note that approximately 90% of the protein is soluble and remains in the initial supernatant, as observed for tapeworm tissue. The calmodulin fraction contained 2.7mg protein, which represents 0.09% of the total protein in the homogenate. Using an alternative method other workers claim to be able to obtain calmodulin samples representing 0.02% of the total protein, so this yield was very good (Means et al. 1991). Analysis of the rat testes calmodulin by SDS-PAGE shows that there is a contaminating protein with a molecular weight of 66kD. See figure 48.

Following this, it was apparent that pig thymus had not necessarily been a good choice of tissue to use to determine the best isolation procedure for tapeworms, as it appeared to have less soluble protein than that found in tapeworm and rat testis. Consequently, all four methods were tried with tapeworm tissue.

Method 1 yielded a tapeworm calmodulin containing fraction, which represented 3% of the homogenate protein, see table 15, which when run on a polyacrylamide gel appeared to be free of contaminating proteins. See figure 49.

When method 2 was tried no protein peak was detected by absorbance at 280nm, on application of the EDTA/EGTA buffer to the phenyl Sepharose column. However, a peak was obtained on application of 6M urea. On analysis by SDS-PAGE (figure 50) a band corresponding to calmodulin can be seen in the 0.5M NaCl wash and to a lesser extent in the application eluent and the regeneration fractions. The protein content of the 0.5M NaCl represented 1.9% of the total protein, which is higher than that obtained in method 1. See table 16.

Sample	Total Protein (mg)	Protein as a percentage of homogenate (%)
Homogenate	2.914g	100
Initial supernatant	2.62g	89
Initial pellet	297	10
Ammonium sulphate precipitation pellet	1.335g	45
Ammonium sulphate isoelectric precipitation supernatant	27	0.9
DEAE Application Eluent	33.25	1.1
DEAE 0.15M NaCl wash	47.5	1.6
Phenyl Sepharose Application Eluent	6.84	0.2
Phenyl Sepharose 0.5M NaCl wash	0.47	0.02
Phenyl Sepharose calmodulin fractions	2.7	0.09
Phenyl Sepharose Regeneration fractions	20.7	0.7

Table 12. Protein Recovery for Calmodulin From Rat Testes

Rat testes was processed according to method 4, to give a comparison with the tapeworm tissue. The rat testes material appears to have a high proportion of soluble protein, like the tapeworm, as shown by the high level of protein in the initial supernatant.

Figure 48. SDS-PAGE of Calmodulin from the Rat Testes

The phenyl Sepharose calmodulin fraction was analysed on a 20% gel. The fraction was found to contain one major contaminating protein of 66kD.

Sample	Protein Content (mg/ml)	Protein Content as a percentage of Homogenate (%)
Homogenate	18.8	100
Initial Supernatant	30	159
Heat Treated Supernatant	1.6	8.5
Heat Treated Pellet	22	117
DEAE Application eluent	3.3	17.5
DEAE 0.15M NaCl wash.	2.9	15.4
Phenyl sepharose Calmodulin fractions	0.65	3.4

Table 15. Protein Recovery of Calmodulin from Tapeworm Using Method 1

Tapeworm tissue was treated using method 1 to extract the calmodulin. Samples were taken throughout the procedure and the percentage protein recovered recorded.

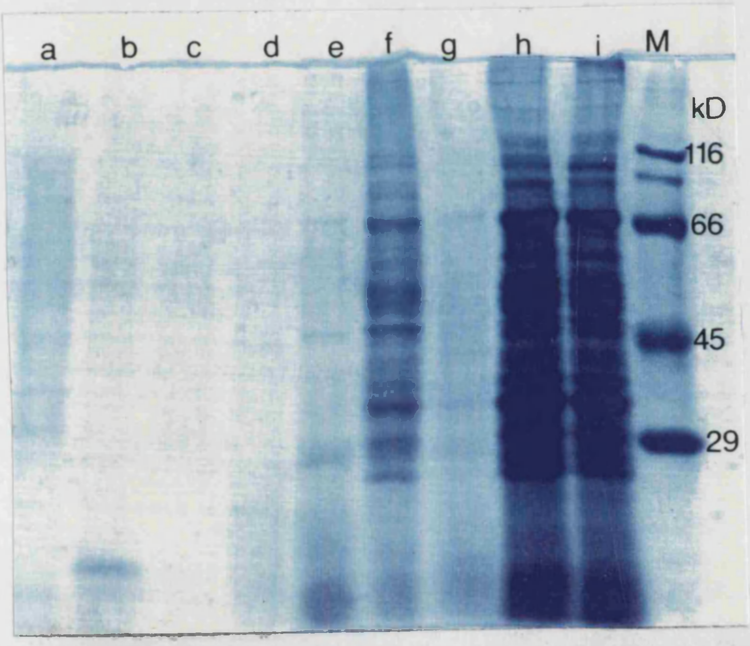


Figure 49. SDS-PAGE of Samples of Tapeworm Using Method 1

Lane a=phenyl Sepharose regeneration fractions, lane b= phenyl Sepharose calmodulin fraction, lane c=blank, lane d=0.15M NaCl DEAE fractions, lane e=DEAE application eluent, lane f and g=heat treated pellet and supernatant, lane h=initial supernatant, lane i=homogenate. M= molecular weight markers.

Sample	Protein Content (mg/ml)	Protein Content as a Percentage of Homogenate (%)
Homogenate	14	100
Resulting resuspended pellet from ammonium sulphate precipitation	3.5	25
DEAE application eluent	4.45	30
DEAE 1.5M NaCl wash	1.5	10
DEAE 0.4M NaCl wash	1.5	10
Phenyl sepharose application eluent	0.38	2.6
Phenyl sepharose 0.5M NaCl wash	0.28	1.9
Phenyl sepharose EDTA/EGTA wash	No protein eluted	-
Phenyl sepharose regeneration	0.5	3.5

Table 16. Protein Recovery of Calmodulin from Tapeworm Using Method 2

Tapeworm tissue was treated as per method 2 to extract calmodulin. Note that no protein was detected in the EDTA/EGTA phenyl Sepharose wash.

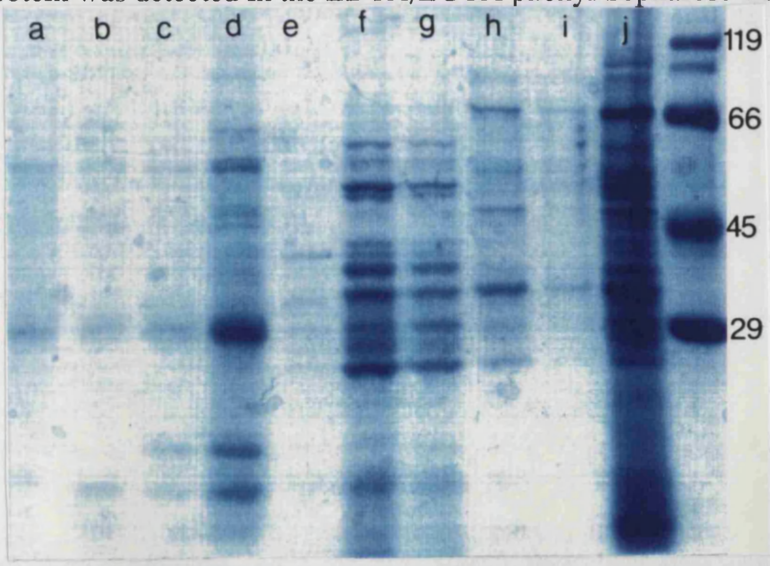


Figure 50. SDS-PAGE Of Samples Of Tapeworm Using Method 2.

Lanes a, b and c= phenyl Sepharose regeneration, 0.5M NaCl wash and application eluent. Lanes d, e, f and g=DEAE 0.5M NaCl wash, 0.15M NaCl wash and application eluent (f and g). Lane h and i= NH_4SO_4 pellet and lane j=homogenate.

Similarly, with method 3 no calmodulin peak was detectable by absorbance at 280nm, on application of the EDTA/EGTA buffer to the phenyl Sepharose column. But, a peak was observed on application of the regeneration solution. On analysis by SDS-PAGE, the regeneration fractions were found to contain a dark band representing calmodulin. See figure 51. It is evident that the calmodulin bound very tightly to the phenyl Sepharose, so requiring the 6M urea used for regeneration to remove it. The protein recovered throughout this method is shown in table 17. The regeneration fraction containing calmodulin had a concentration of 1.9mg/ml, which is equivalent to 7.5% of the total protein. This is significantly more than that obtained with either method one or two.

Sample	Protein Content (mg/ml)	Protein content as a percentage of homogenate
Homogenate	19.95	100
Initial supernatant	17.25	86
Initial pellet	2.7	13
Resuspended TCA pellet	1.73	8.6
DEAE application eluent	12	60
DEAE 0.15M NaCl wash	0.4	2
Phenyl sepharose application eluent	1.98	9.8
Phenyl sepharose EDTA/EGTA wash	No peak	-
Phenyl sepharose regeneration	1.9	7.5

Table 17. Protein Recovery for Tapeworm Using Method 3

Tapeworms were treated as per method 3 to isolate calmodulin. On application of the EDTA/EGTA buffer to the phenyl Sepharose column, no peak formed and no protein was detected in the eluent. However, a peak did form on application of 6M urea.

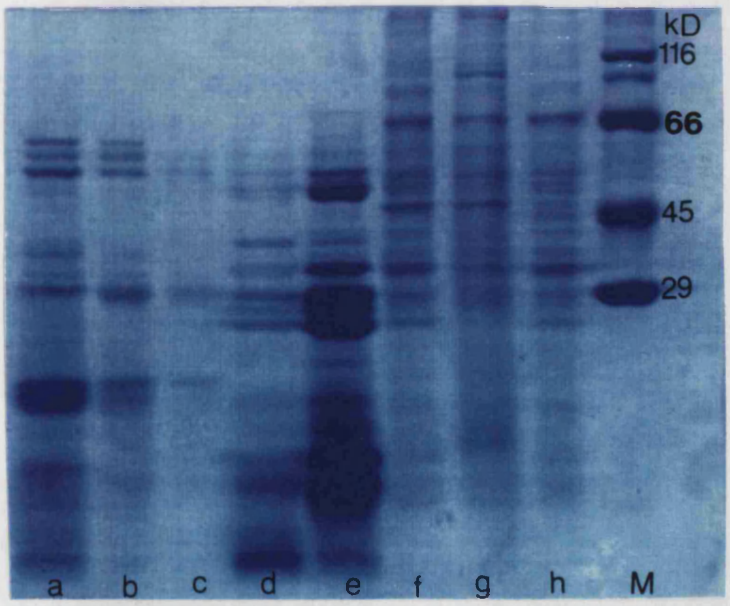


Figure 51. SDS-PAGE of Samples Of Tapeworm Using Method 3

Lane a=phenyl Sepharose regeneration, lane b and c=phenyl Sepharose application eluent, lane d and e=DEAE application eluent, lane f and g=initial pellet, lane h=initial supernatant. M=molecular weight markers.

3.2.2 Phosphodiesterase Experiments

As already stated in section 2.3.4., phosphodiesterase was used as an assay system to test the biological activity of the isolated samples of calmodulin. Initially some experiments were performed using some commercial calmodulin to establish the optimum conditions for this coupled assay system.

The formula used to calculate the specific activity in all of the phosphodiesterase experiments was as follows:

Total Reaction (TR) =

$$\frac{\text{counts per minute - background counts per minute}}{\text{sample volume}} \times \text{total reaction volume}$$

Total Counts (TC) =

$$\frac{\text{total counts per minute - background counts per minute}}{\text{number of Moles of cAMP.}}$$

and $$\frac{TR}{TC} = \text{cpm per Mole of cAMP hydrolysed}$$

where 1 ^{3}H-adenosine is equivalent to 1 ^{3}H AMP and therefore 1^{3}H cAMP.

and finally:

$$\text{Specific activity} = \frac{(TR/TC)}{\text{mU phosphodiesterase}}$$

$$= \text{mols/ minutes/mU phosphodiesterase.}$$

(For definition of units see appendix 1)

One of the first experiments was to determine whether to use the Dowex ion exchange resin in a batchwise method or as a mini-column (1ml) as both have been described in the literature. Reaction vessels were set up in triplicate containing either a)phosphodiesterase and substrate, b)substrate alone, and c)phosphodiesterase, substrate and calmodulin (as detailed in section 2.3.4.). All were incubated for 10 minutes at 30°C. Better results were obtained using the batch method than the mini-columns, there being approximately 500 counts per minute difference between the two. The batchwise system was also much quicker to perform than the mini-columns, so that for larger scale experiments the mini-columns would prove impractical.

In this first experiment it was also found that the commercial calmodulin did not appear to stimulate the enzymes activity. When the mini-column system was used, calmodulin appeared to decrease it from 4nmols cAMP/minute to 1nmol cAMP/minute. But the batchwise results showed no difference, yielding 4nmols cAMP/minute with or without calmodulin.

A larger experiment was performed to determine the optimum incubation times and conditions. The results can be seen in figure 52. It was found that the peak rate of hydrolysis occurred when the reaction mixture was incubated for 10 minutes. The inclusion of calmodulin in the reaction mixture appeared to increase the activity by almost 50% to 2.3nmols/minute/mU PDE.

Following this, two separate experiments were performed, one to establish the optimum quantity of calmodulin to stimulate phosphodiesterase and the other to investigate the effect of incubation time on the rate of reaction. The results are shown in figure 53. It can be seen in figure 53A that there is a steady increase in the rate of hydrolysis of cAMP, on the addition of between 5 and 15 units of commercial calmodulin. However, the addition of 20U of Sigma calmodulin appears to have decreased the rate of activity to that obtained by the addition of 10U calmodulin.

Pig thymus calmodulin, obtained by either method 1 or method 4, increased the rate of hydrolysation of cAMP. The calmodulin obtained using method 4 had a much greater effect increasing the rate from 0.6nmols/minute/mU to 1.66nmols/minute/mU whereas calmodulin from method 1 only increased the rate to 0.82nmols/minute/mU.

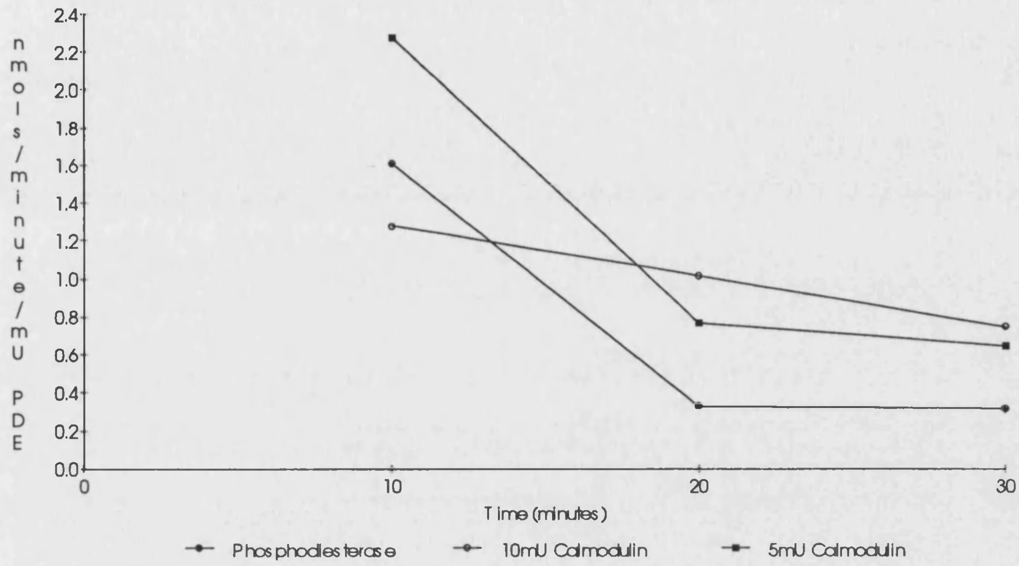


Figure 52. Optimisation Experiment For Phosphodiesterase Assay

This shows the specific activity for phosphodiesterase where the rate of hydrolysis of cAMP was determined over 30 minutes both in the presence and absence of calmodulin. (Representative graph from one experiment, performed in triplicate, using mean data with error margin of ±50cpm).

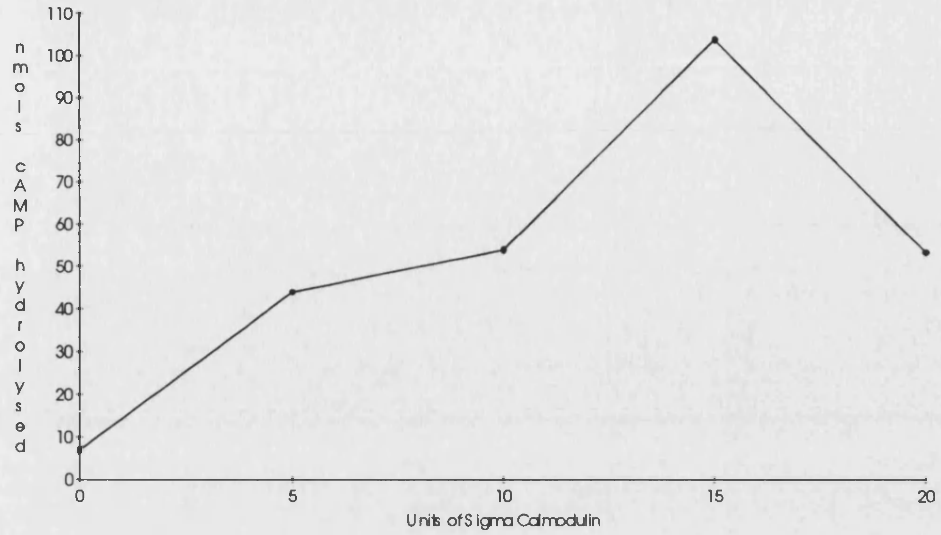


Figure 53A. Effect of Commercial Calmodulin on Phosphodiesterase

This shows that up to 15 units of Sigma calmodulin stimulates phosphodiesterase. However there is a reduced level of activation when 20 units of calmodulin is added.

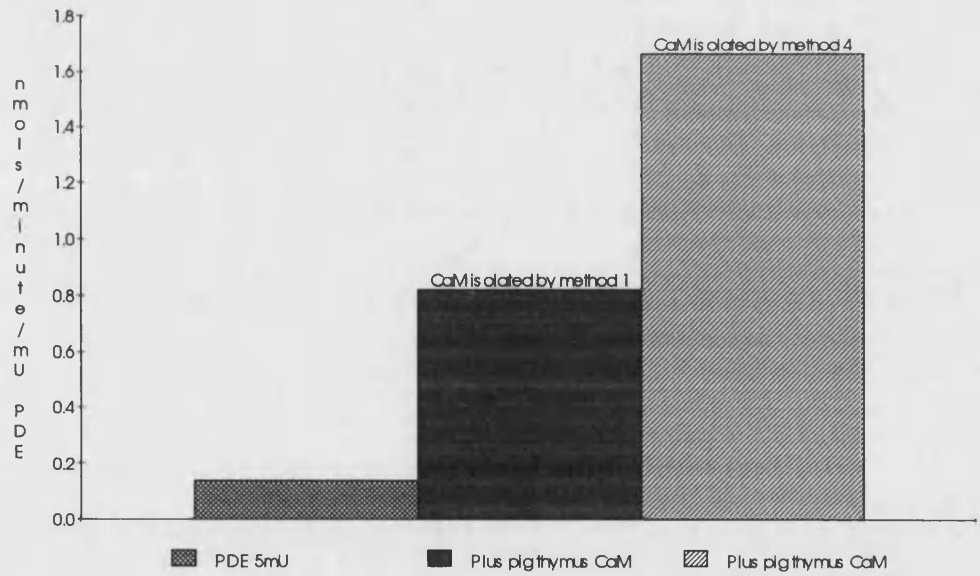


Figure 53B. Effect of Pig Thymus Calmodulin on Phosphodiesterase

It can be seen that the presence of pig thymus calmodulin (CaM) increases the rate of hydrolysis of cAMP by phosphodiesterase. The calmodulin obtained by method 4 appears to be the most stimulatory.

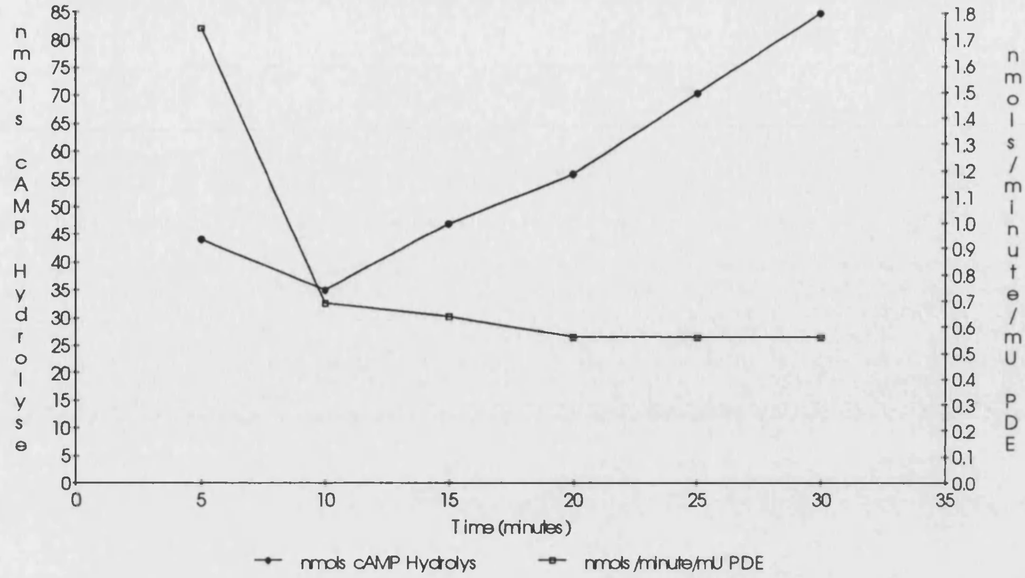


Figure 53C. Time Course Experiment for Phosphodiesterase

This shows that after 10 minutes incubation there is a steady increase in the number of moles of cAMP hydrolysed, together with a steady specific activity of around 0.55 nmols/minute/mU PDE. (Graph shows mean data from a representative experiment performed in triplicate. Error margin on original data= ±42cpm).

This is particularly noteworthy as there were 48μg of method 1 calmodulin and only 7.5μg of method 4 calmodulin. This could mean that the calmodulin obtained by method 1 had undergone some minor modification, possibly by the action of proteases. It could also mean that the calmodulin contained some inhibitory substance. However, it should be remembered that the 5U of Sigma calmodulin gave the same level of activation, i.e. 0.8nmols/min/mU phosphodiesterase.

The time course experiment showed that after 10 minutes there was virtually a linear increase in the number of moles of cAMP hydrolysed, with a relatively constant specific activity with a mean value of 0.6+/-0.09nmols/minute/mU phosphodiesterase. However, this is lower than the specific activity determined in the previous optimisation reaction where the specific activity was 1.3nmols/minute/mU PDE. Consequently an experiment was conducted to determine the effects of the tapeworm calmodulin.

In this experiment it was found that the basal activity of the phosphodiesterase was the same as that found in the first experiment, 1.34nmols/min/mU phosphodiesterase. See figure 54. However, there was a decreased response to the Sigma calmodulin compared to previous experiments, with only the presence of 10U calmodulin being stimulatory. The tapeworm calmodulin also failed to activate the phosphodiesterase above its basal level.

It was apparent that there was an inconsistency in the observed basal activity of the phosphodiesterase in these different experiments. Consequently, to check that the problem was not due to degradation of the ^{3}H-cAMP, a sample of the ^{3}H-cAMP was analysed by thin layer chromatography as described in section 2.3.5. The results can be seen in figure 55.

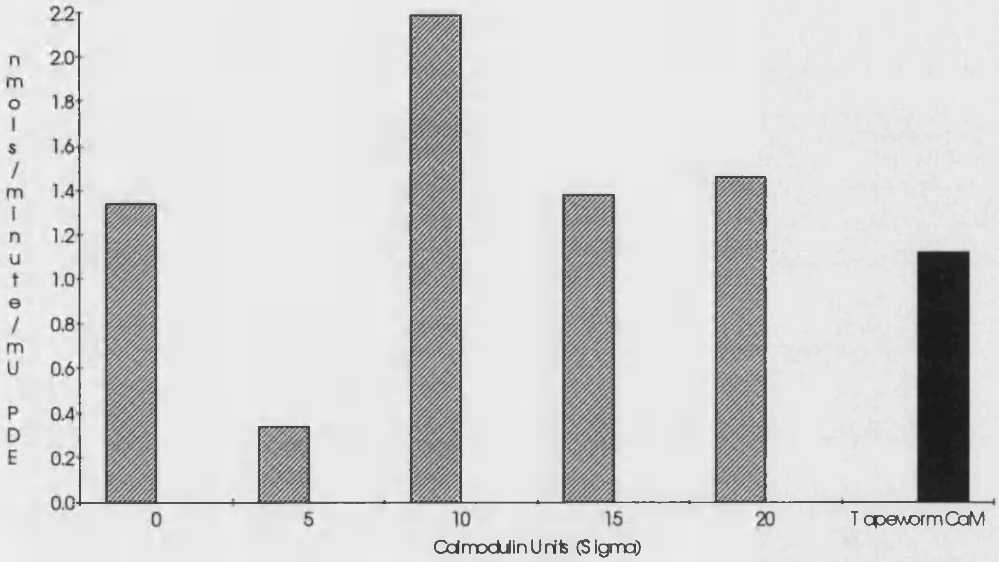


Figure 54. Effect of Calmodulin on Activity of Phosphodiesterase

On this occasion both calmodulin from Sigma and that isolated from tapeworms were used in the experiment. Only the addition of 10 units of commercial calmodulin appears to stimulate phosphodiesterase. (Graph shows mean data, error margin on original data ±97cpm).

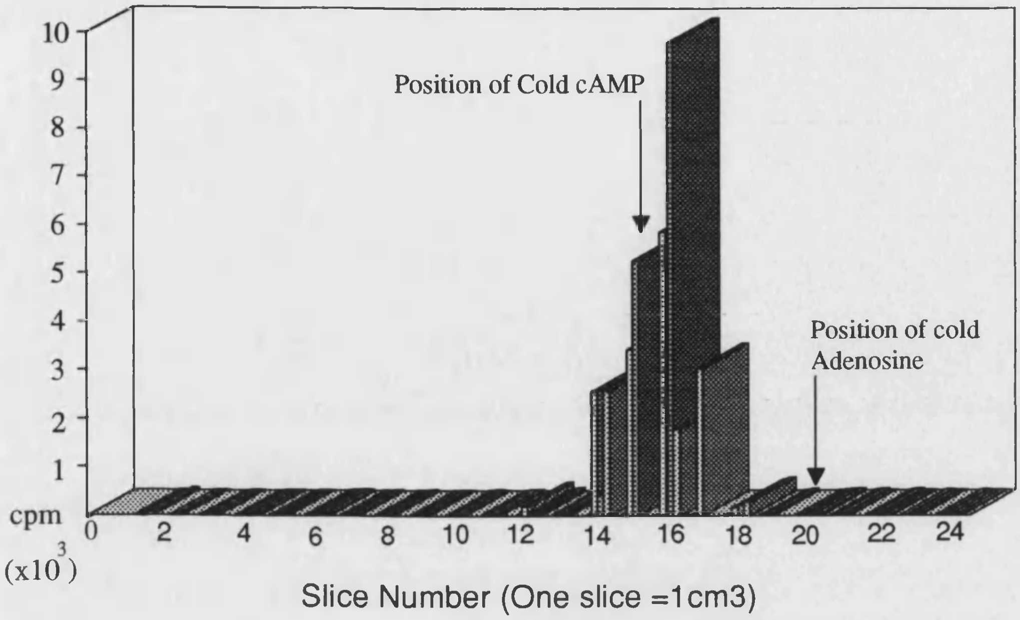


Figure 55. Thin Layer Chromatography of cAMP

Samples of cAMP, adenosine and ^{3}H-c-3',5'-AMP were run on a thin layer chromatogram. The positions of the cold adenosine and cAMP were noted under UV light prior to the chromatogram being cut into 1cm^3 slices which were counted to reveal the position of the cAMP to determine if any degradation had occurred. The equivalent positions of the cold adenosine and cAMP are marked, from which it can be seen that the ^{3}H-cAMP does not contain any adenosine. (cpm= counts per minute, cAMP= cyclic adenosine monophosphate).

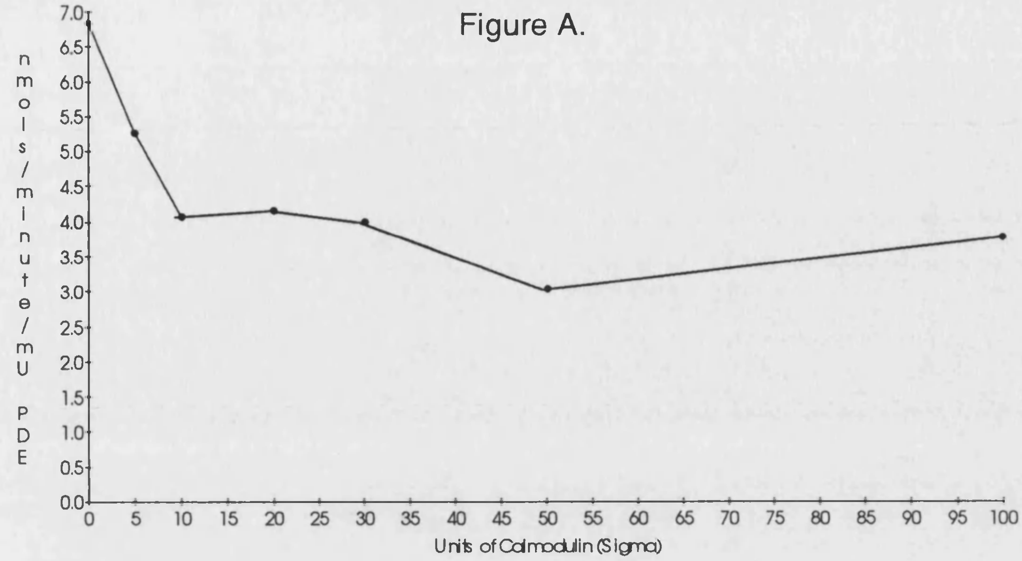
It can be seen that the ^{3}H-cAMP forms a single peak of radioactivity corresponding to the position of the non-radiolabelled c-AMP marker. There is no radioactivity corresponding to the position of the adenosine marker. The Rf values were calculated, where:

$$Rf = \frac{\text{distance protein migrated from base line}}{\text{distance from base line to solvent front.}}$$

and were found to be 0.76 for adenosine, and 0.675+/- 0.015 for cAMP. Values above 0.2 indicate that full separation of the samples should have occurred (Randerath & Randerath 1967). Further thin layer chromatograms were performed throughout the work to check the state of the c-^{3}H-AMP. In all cases the results obtained were the same as those reported here with the Rf values being between 0.45 and 0.80.

Due to the disappointing results obtained in the previous experiment with calmodulin and phosphodiesterase, the experiment was repeated again using different quantities of pig thymus, tapeworm and commercial samples of calmodulin. The results are shown in figure 56. It was found that the basal activity of the phosphodiesterase had increased to 6.84+/-1.04nmols/minute/mU phosphodiesterase from the 1.34nmols/minute/mU, found in the previous experiments. Therefore it was unsurprising to find that the calmodulin samples all failed to stimulate the phosphodiesterase and in fact appeared to inhibit the reaction.

To further test this finding two reaction vessels were prepared containing sufficient reaction mixture to take samples at intervals during one hour. The results show that there is little if any response to the calmodulin apart from what appears to be a rogue value at 40 minutes. See figure 57.



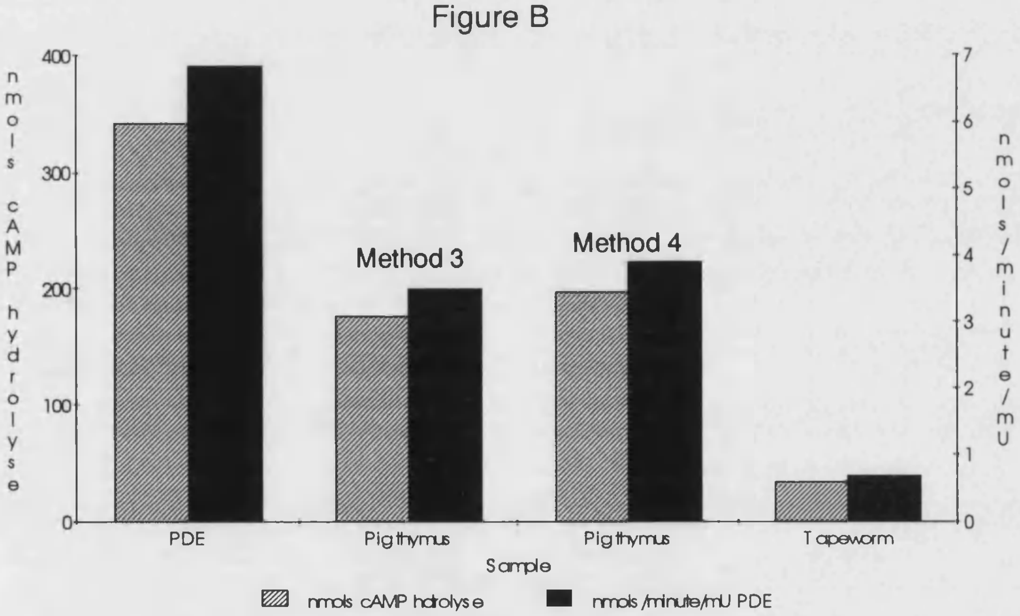


Figure 56. Repeat Experiment of the Effects of Calmodulin on Phosphodiesterase Activity

Following the check on the ^{3}H-cAMP, the experiment to test the effect of calmodulin on the rate of hydrolysis of cAMP was performed. Figure A shows the effects of different quantities of Sigma calmodulin. Figure B shows the effect of calmodulin isolated from pig thymus and tapeworm. (Error on original data ±75cpm).

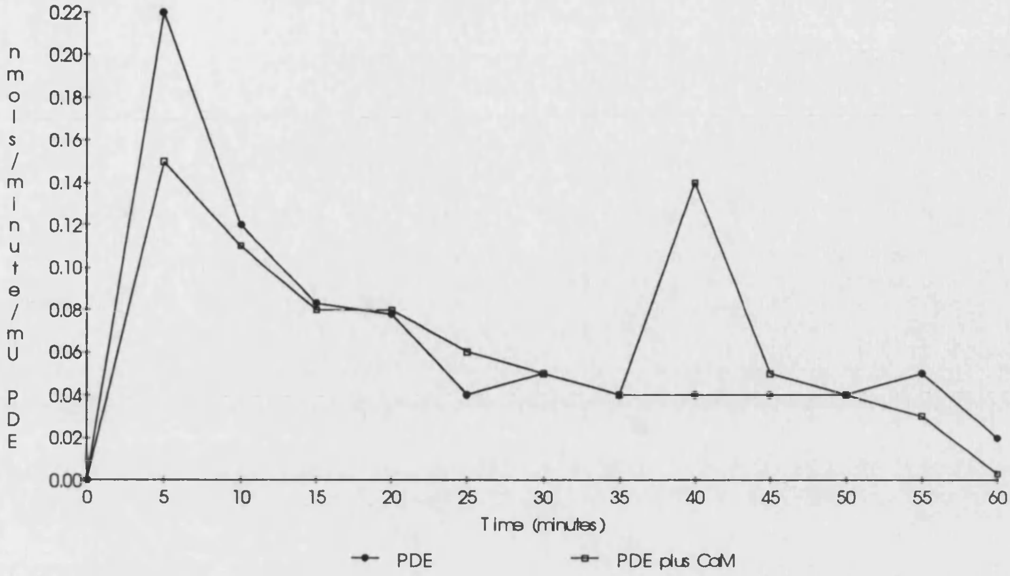


Figure 57. Continuous Time Course Experiment for Phosphodiesterase

Two phosphodiesterase reaction vessels were set up one with calmodulin and the other without. The tubes were incubated at 30°C for one hour with samples being taken every 5 minutes. (PDE = phosphodiesterase, CaM= calmodulin).

It was apparent that the reaction was not proceeding as expected, as the phosphodiesterase's basal activity was increasing with a concomitant decrease in sensitivity to samples of calmodulin. It was then discovered from a manufacturer of phosphodiesterase (Pharmacia) that once the lyophilised phosphodiesterase is prepared in a solution it becomes unstable and is only sensitive to calmodulin in the first six hours. After that time, its own basal activity increases, with the simultaneous loss of sensitivity to the stimulatory effects of calmodulin. The phosphodiesterase that had been used in the above experiments had been prepared into a solution at the start of these experiments and then stored at 4°C until the next experiment was performed. Consequently the results obtained appear to support the opinion that phosphodiesterase becomes unstable over time. This also means that phosphodiesterase is not a very good candidate for an assay system to test the biological activity of calmodulin.

3.2.3 Phosphodiesterase Calmodulin Sensitivity Experiment

To test the hypothesis that phosphodiesterase's basal activity was increasing over time, with a decrease in calmodulin sensitivity the following experiment was designed. The principle of this experiment is that there should be a reduction in the time with which phosphodiesterase will remain 'bound' to the calmodulin on a calmodulin-agarose column, the longer the phosphodiesterase has been prepared from its lyophilised state before application to the column. The results are shown in figures 58 A to E. These were calculated using the formulae given earlier for calculating the number of moles of adenosine produced, section 3.2.2. At time=0, figure A, there is a small amount of activity washed off in fractions 12-17 indicating

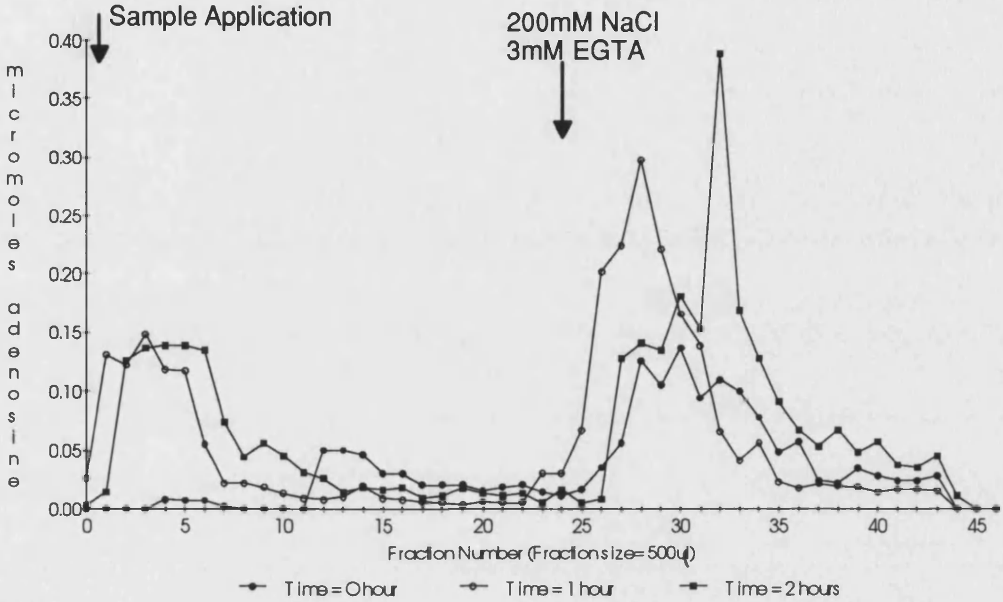


Figure 58A. Phosphodiesterase Sensitivity Experiment Hours 0-2

The reactivity found in each fraction, eluted from the calmodulin column, is expressed as the number of micromoles of adenosine which is equivalent to the number of micromoles of cAMP hydrolysed by phosphodiesterase.

that not all the phosphodiesterase bound to the column. The phosphodiesterase was then eluted from fractions 27 to 37. After the first hour (figure 58B) there is far more phosphodiesterase that is not binding to the column, with activity being found in fractions 2 to 10. However, the remaining activity was eluted in fractions 25-37. This pattern of elution remains relatively constant for up to 6 hours. Then the bound phosphodiesterase starts to elute slightly earlier between fractions 26 and 35, and from 8 hours onwards the activity is eluted in fractions 22 to 35 (figure 58 C & D). After 12 hours the phosphodiesterase is eluted somewhat erratically from fractions 14 to 23. At 24 hours there are no discernible peaks of activity in the fractions, figure 58E. The pattern of elution is very random, and implies that there is no longer specific interaction with the calmodulin coupled on the agarose. The results show that as time progresses, there does appear to be less interaction between calmodulin and phosphodiesterase so supporting the hypothesis.

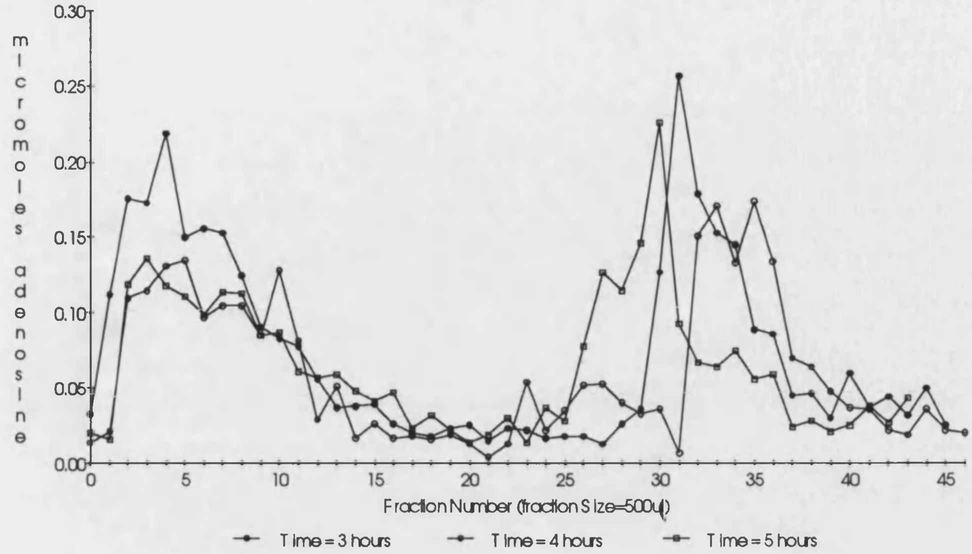


Figure 58B. Phosphodiesterase Sensitivity Experiment Hours 3-5

This shows the reactivity found in fractions eluted from calmodulin agarose columns used for time points between 3-5 hours.

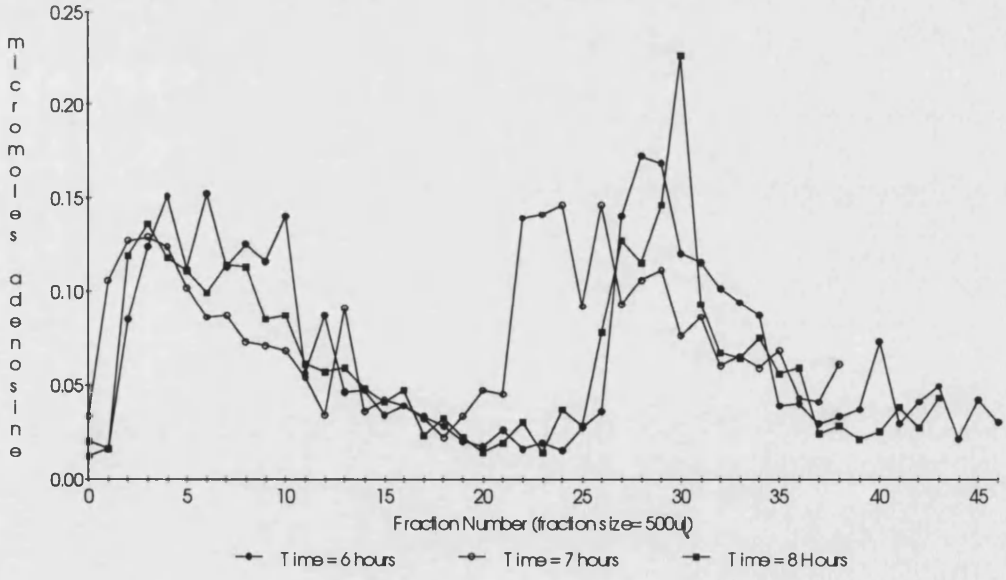


Figure 58C. Phosphodiesterase Sensitivity Experiment Hours 6-8

This shows the reactivity in fractions eluted from calmodulin agarose columns used for hours 6, 7 and 8.

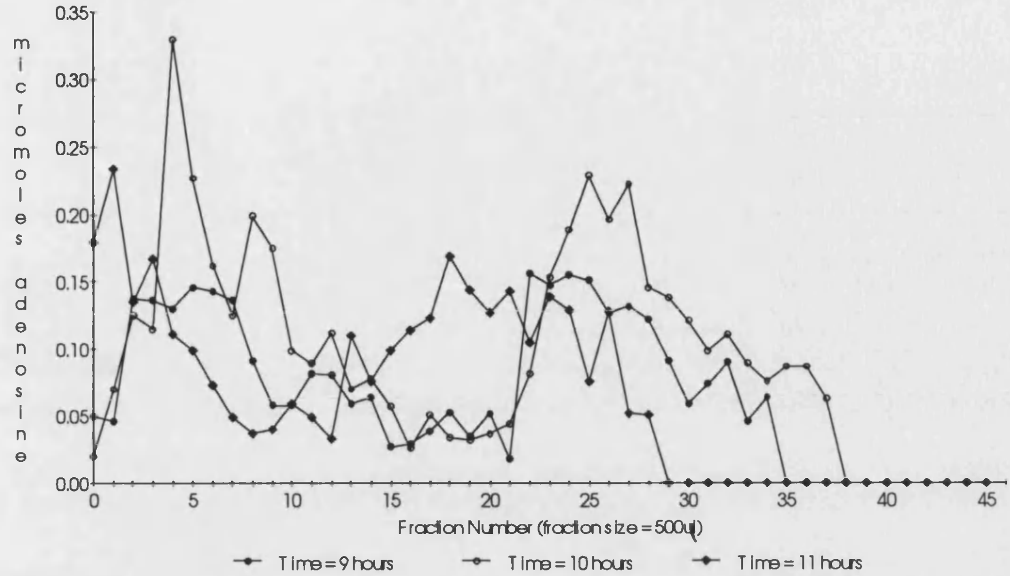


Figure 58 D. Phosphodiesterase Sensitivity Experiment Hours 9-11

This shows the reactivity of fractions obtained from columns for hours 9-11.

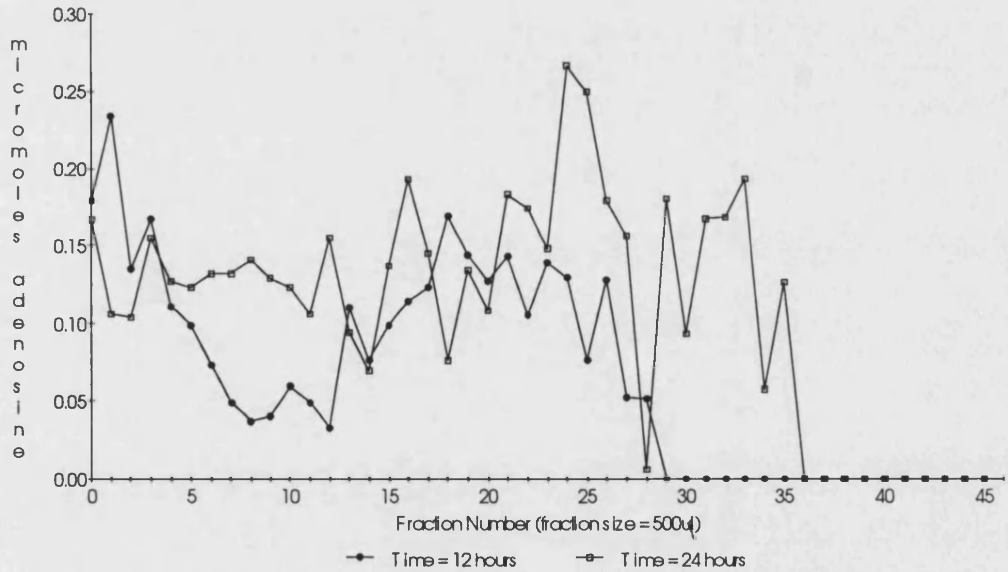


Figure 58E. Phosphodiesterase Sensitivity Experiment Hours 12-24

This shows the reactivity found in fractions obtained from columns used for 12 and 24 hours.

3.2.4 Immunogenic Recognition of Isolated Calmodulin.

Two other approaches were used to analyse the calmodulin samples, which would demonstrate that the calmodulin had retained its antigenicity and was indeed calmodulin. These were an ELISA (enzyme linked immunosorbant assay) and a western blot. Initially an ELISA was performed (details in section 2.3.8) to establish if the anti-calmodulin antibody recognised anything in the isolated calmodulin fractions and to determine the optimum concentration of calmodulin antibody to use in western blots. The results obtained for pig thymus and commercial calmodulin are shown in figure 59. The graph shows that there is a concentration dependent decrease in the absorbance at 450nm, as expected and that the optimum dilution of antibody for Western blot is 1-500. Similar results were obtained for the tapeworm calmodulin obtained using the three different methods of isolation. The results are shown in figure 60. It can be seen that the Sigma calmodulin does not follow the same pattern of curve on this occasion. A further ELISA of the Sigma calmodulin was performed where it was found to have a normal concentration dependent curve.

Initial Western blots showed a positive hybridization to a protein band with a molecular weight of 66kD, in both pig thymus and tapeworm calmodulin, but not in the commercial calmodulin. This could represent calmodulin bound to one of its target proteins or it could be a protein in which calmodulin forms a permanent regulatory domain. Two possible proteins which have subunits with a molecular weight of around 60kD are the multifunctional protein kinase, 49-63kD, (section

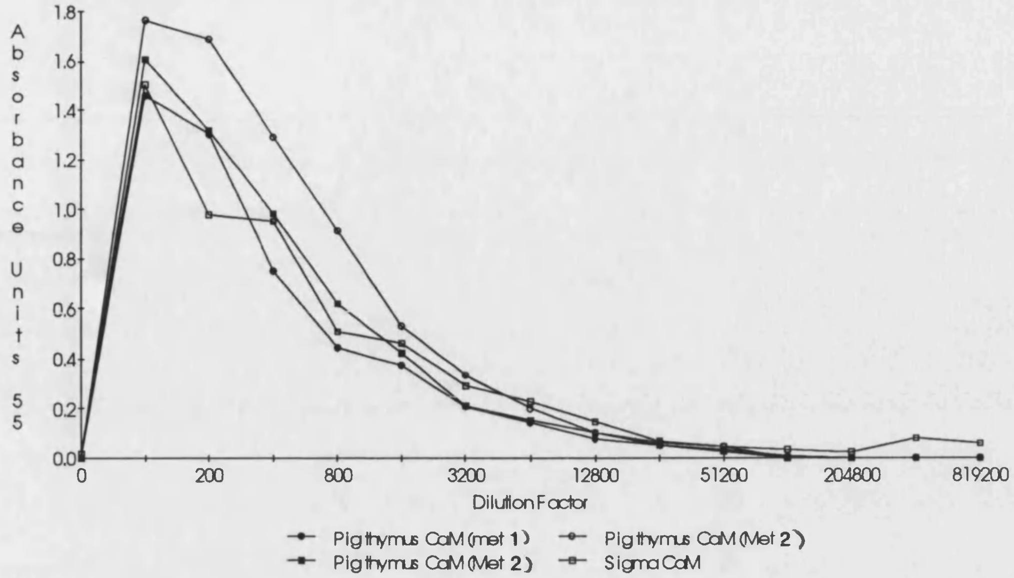


Figure 59. ELISA of Calmodulin Fractions From Pig Thymus

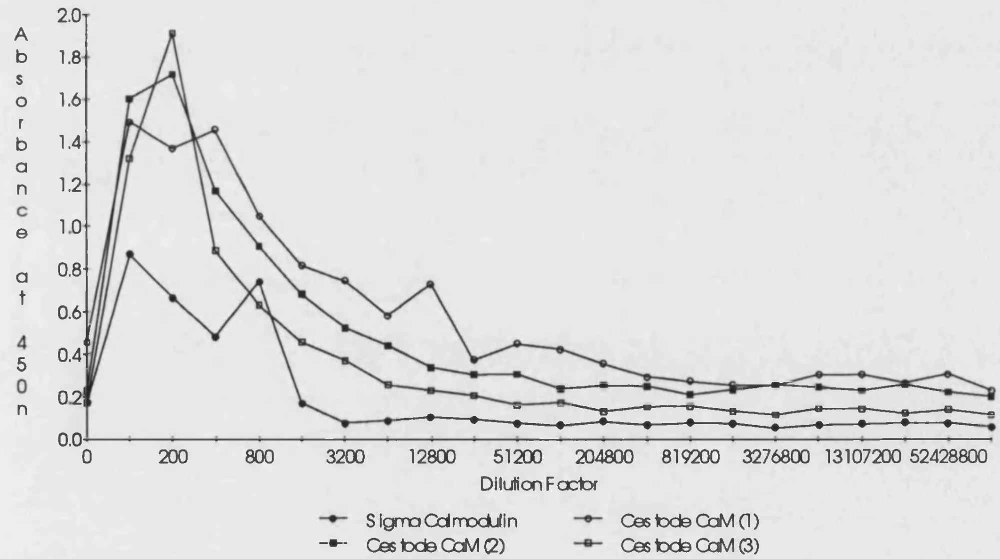


Figure 60. ELISA of Calmodulin Fractions from Tapeworm

Calmodulin samples obtained from each of the different methods were treated. The isolation method used to obtain the calmodulin is shown in brackets in the legend. (CaM = calmodulin).

1.1.1.1.2.) and phosphodiesterase, 57-60kD, (section 1.1.1.1.4.). It seems unlikely that it is phosphodiesterase as if this were present in the calmodulin samples then the results from the above phosphodiesterase experiments should have been affected. But, it could quite feasibly be a calmodulin multifunctional protein kinase. Western blot analysis of the rat testis initial supernatant revealed antibody binding to two bands' one at 66kD and another with a molecular weight of around 45kD.

The western Blot of the tapeworm calmodulin can be seen in figure 61. In all cases a band representing calmodulin was formed by the antibody. Interestingly, though, in method 2 two other proteins were also highlighted, forming bands of around 36kD. When the initial homogenate was probed with antibody several bands gave positive hybridisation with the calmodulin antibodies.

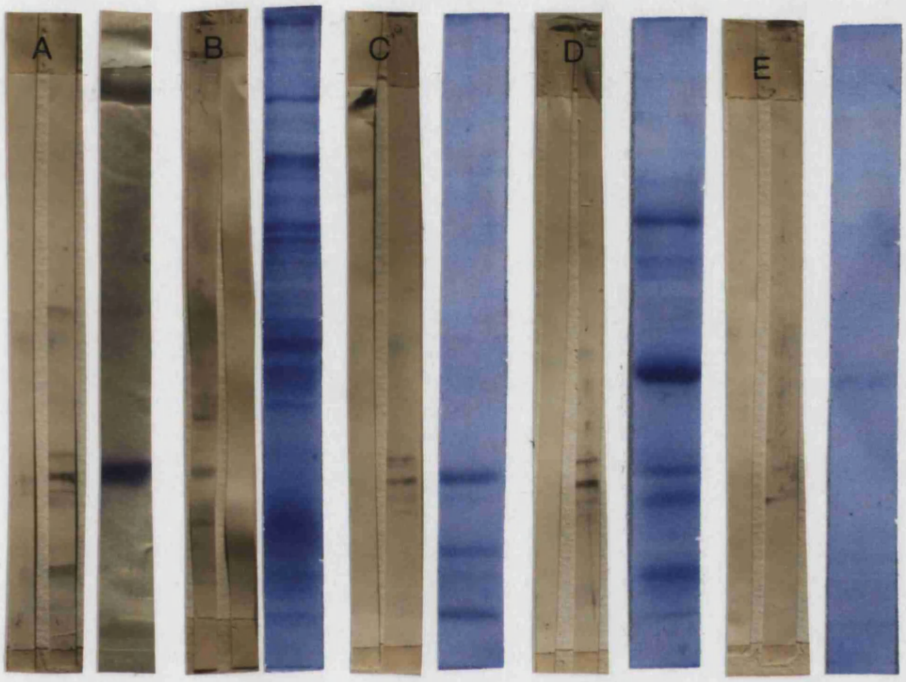


Figure 61. Western Blot of Isolated Tapeworm Calmodulin Fractions

Each figure shows the Western Blot and the duplicate SDS-PAGE. Figure A= Sigma calmodulin. Figure B=tapeworm homogenate, figure C=method one tapeworm calmodulin, figure D=method 2 tapeworm calmodulin, figure E=method 3 tapeworm calmodulin.

3.2.5 Isolation of Calmodulin Binding Proteins

A number of different conditions were used to investigate the tapeworm calmodulin binding proteins. Initially a column was run where the tapeworm tissue was treated both with and without the addition of detergent. It was thought that the inclusion of 3.5% (v/v) Triton X-100 would solubilise any membrane bound calmodulin binding proteins. The elution profile for each of these is shown in figure 62.

SDS-PAGE revealed that tapeworm homogenate treated with detergent contained more calmodulin binding proteins than the homogenate treated without detergent. See figure 63. Calmodulin binding proteins from the homogenate, not treated with detergent, had the following molecular weights: one of 66kD, four proteins with molecular weights between 45kD and 66kD, one of 45kD, one with a molecular weight between 36kD and 45kD which forms a feint band, one of 36kD which also forms a feint band, one of just under 36kD and one with a molecular weight between 24 and 29kD. Whilst the calmodulin binding proteins isolated from the homogenate treated with Triton had the following molecular weights: one of 205 kD, one between 205kD and 116kD, one of 116kD, one of 97kD, one of 66kD three with molecular weights between 66kD and 97kD, one of 45kD, two possibly three with molecular weights of between 29kD and 45kD, and one of approximately 20kD. In both cases there are proteins, which form a band smaller than 20kD.

In both of the above columns, the homogenate was prepared in a calcium containing buffer and applied to the calmodulin-agarose column. The presence of the calcium theoretically enables the calmodulin binding proteins to interact with the calmodulin. These proteins can then be eluted by applying a buffer containing EDTA or EGTA.

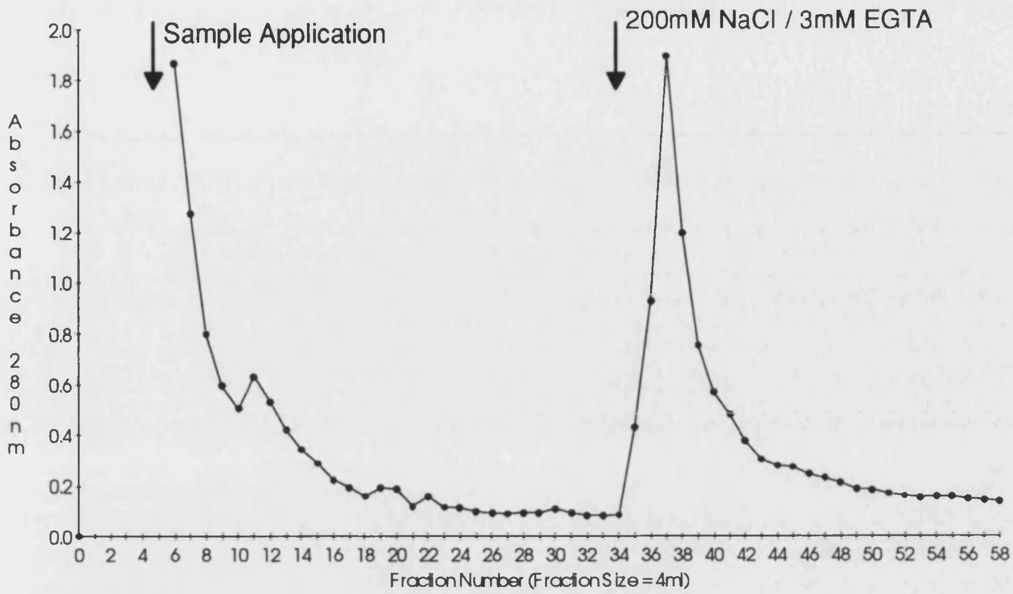


Figure A

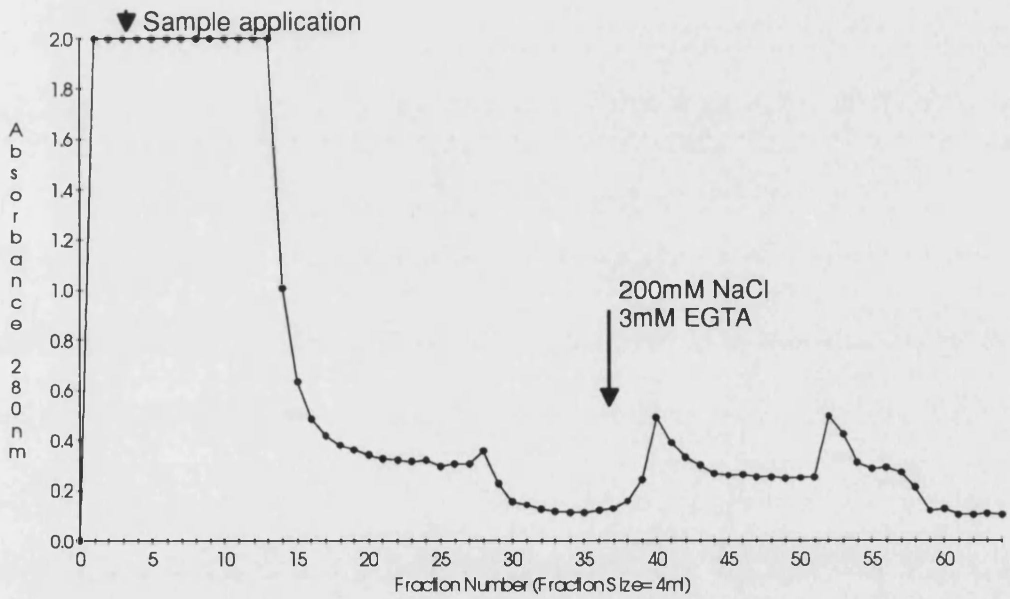


Figure B.

Figure 62. Elution Profile for Calmodulin Agarose

Figure A shows the elution profile for tapeworm tissue **not** treated with detergent.

Figure B shows the elution profile for tapeworm tissue treated with detergent.

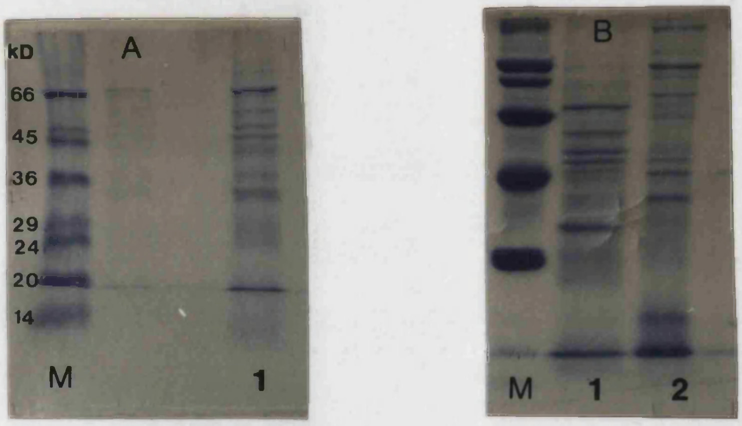


Figure 63. SDS-PAGE of Calmodulin Binding Proteins Isolated From

H.diminuta

Figure A. Lane 1 shows the calcium dependent calmodulin binding proteins isolated without detergent. M=molecular weight markers. Figure B. Lane 1=calmodulin agarose wash with detergent, lane 2=calcium dependent calmodulin binding proteins isolated with detergent. M=molecular weight markers:-205, 116, 97, 66, 45 and 29kD.

A column was run to test that these proteins were actually eluting due to the chelation of calcium ions by EGTA. This entailed homogenising the tissue in a calcium free buffer and applying it to a calmodulin-agarose column. The proteins were then eluted with an EGTA buffer containing 200mM NaCl. Theoretically no proteins would be expected to bind to the calmodulin agarose, due to the absence of calcium. However, it was found that on application of the elution buffer, proteins did elute forming a peak. See figure 64. The fractions forming the peak were pooled and analysed by SDS-PAGE, the results of which can be seen in figure 65. The peak obtained from the EGTA column shows only three proteins with molecular weights of 205kD, 97kD and 45kD.

Having established that some protein bound to the column in a calcium independent manner, and was eluted by an increase in the salt concentration, another column was run with no salt gradient. The tissue was homogenised in buffer containing calcium and 50mM NaCl and applied to the column. The calmodulin binding proteins were eluted with a buffer containing EGTA and 50mM NaCl. Further a second wash was performed using an EGTA buffer containing 200mM NaCl as used in the first columns. Interestingly a peak formed with both elution buffers. See figure 66.

After having performed these two sets of columns, i.e. one with calcium and the other without calcium, it became apparent that there were proteins present in the cestode, which both bind with calmodulin in a calcium dependent and independent manner. However, to make sure that the presence of $MgCl_2$ in the buffer systems was not affecting the binding a column was prepared using homogenising buffer containing EGTA and 3mM $MgCl_2$. The column was then washed with the same buffer containing 1mM $MgCl_2$.

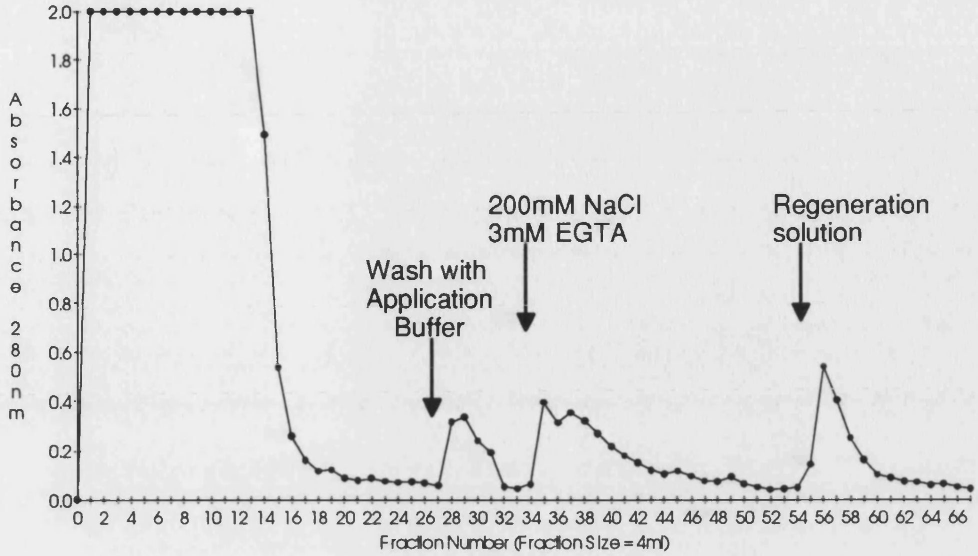


Figure 64. Elution Profile for Isolation of Calcium Independent Binding Proteins

EGTA was included in all buffers to ensure that only proteins which bind to calmodulin in a calcium independent fashion were isolated.

Figure 65. SDS-PAGE of Calmodulin Binding Proteins

Lanes a, b,c and d=calcium treated tissue. Lanes a and b=initial supernatant and pellet, lane c=application eluent, lane d=calcium dependent calmodulin binding proteins. Lanes e to h =EGTA treated tissue. Lane e and f=initial supernatant and pellet, lane g=application eluent, lane h=calcium independent calmodulin binding proteins.

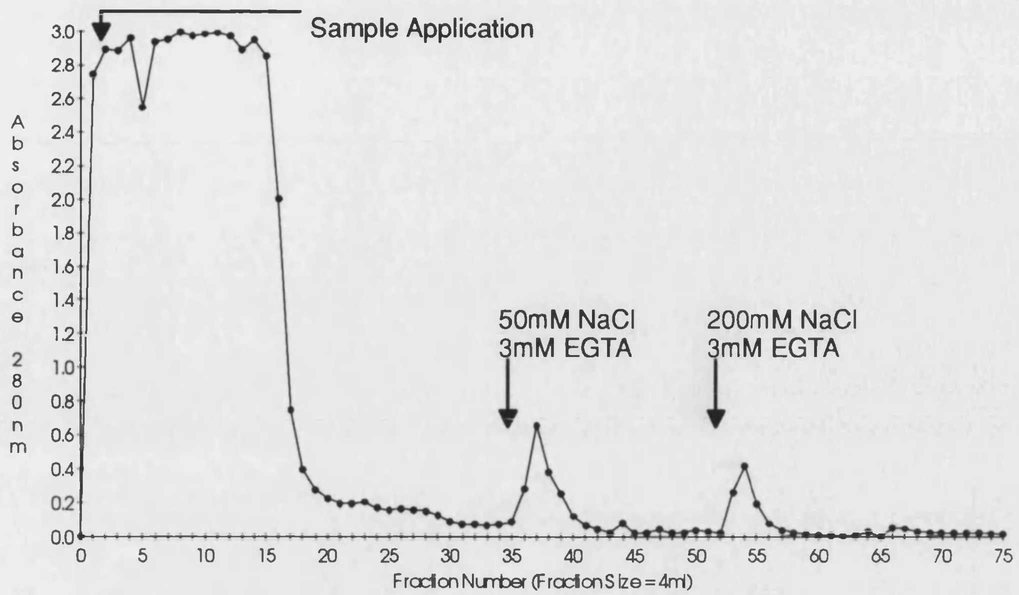


Figure 66. Elution Profile for Calmodulin Agarose Run with a Step Salt gradient

Tapeworm homogenate was applied to a calmodulin agarose column in the presence of calcium, the calmodulin binding proteins were then eluted first by the addition of an EGTA buffer, and then by an EGTA buffer containing 200mM NaCl.

Finally the column was washed with buffer containing EGTA, 1mM $MgCl_2$ and 200mM NaCl. On application of each buffer a peak eluted. This was somewhat surprising. Figure 67A and B show the elution profile together with the SDS-PAGE of the fractions.

During these experiments it had been noticed that the column appeared to be aggregating, forming dense white clumps, and that the flow rate had gradually diminished. On viewing a sample of the calmodulin-agarose under a light microscope it was found that the agarose beads had indeed aggregated. See figure 68. Consequently a new batch of calmodulin-agarose was dispensed, and all the above experiments repeated. A procedure was developed to remove any residual material from the calmodulin-agarose between column runs to prevent any aggregation of the new agarose gel.

After the column had been regenerated the column was unpacked and the agarose placed into a Falcon tube, to which 5 volumes of regeneration buffer containing 0.1% non-ionic detergent which in this case was Triton X-100. The mixture was then placed at 37°C for 1 minute with agitation. Following this the mixture was washed three times with regeneration buffer to remove any residual detergent. Finally the column was re-packed and pre-equilibriated.

Initially a column was run with calcium and a step salt gradient to isolate calcium dependent calmodulin binding proteins. The elution profile is shown in figure 69. Then a column without calcium was run to isolate any calcium independent calmodulin binding proteins. It was found that the calcium independent calmodulin binding proteins eluted with buffer containing 200mM NaCl and not with 50mM NaCl as seen for calcium dependent calmodulin binding proteins. See figure 70.

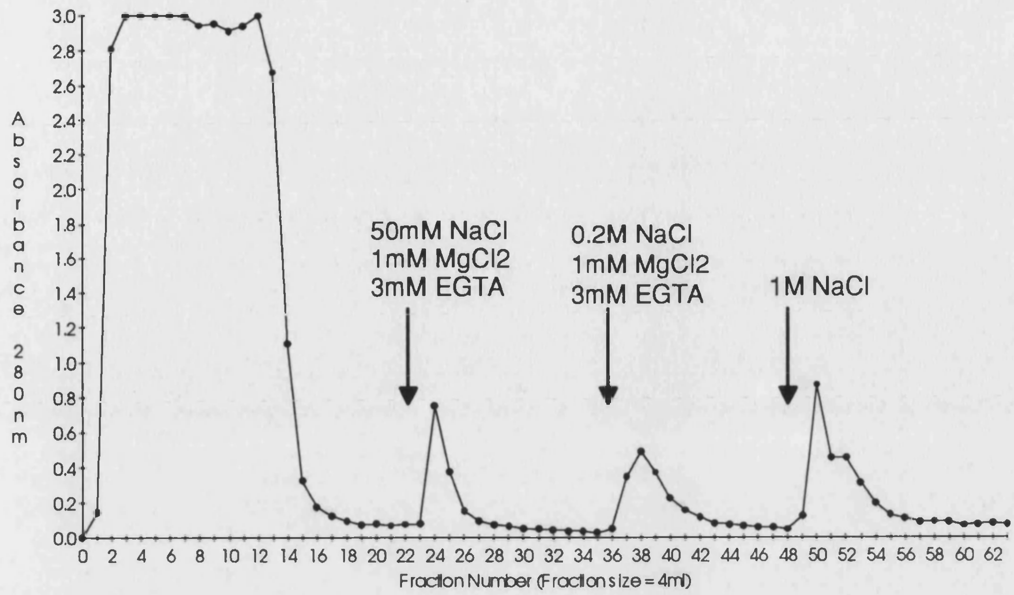


Figure 67A Elution Profile for Calmodulin Agarose Run Stepwise

The calmodulin agarose was run in a sequential matter, using a stepwise salt gradient.

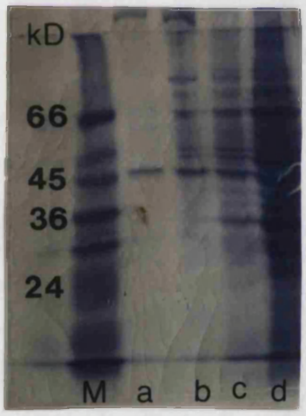


Figure 67B. SDS-PAGE of Calmodulin Binding Proteins Isolated in Figure 65A

Lane a=regeneration fractions (1M NaCl), lane b=calmodulin binding proteins eluted with 0.2M NaCl, lane c=proteins eluted with change in $MgCl_2$ concentration. M=molecular weight markers, 66, 45, 36, 29, 24, 20 and 14kD.

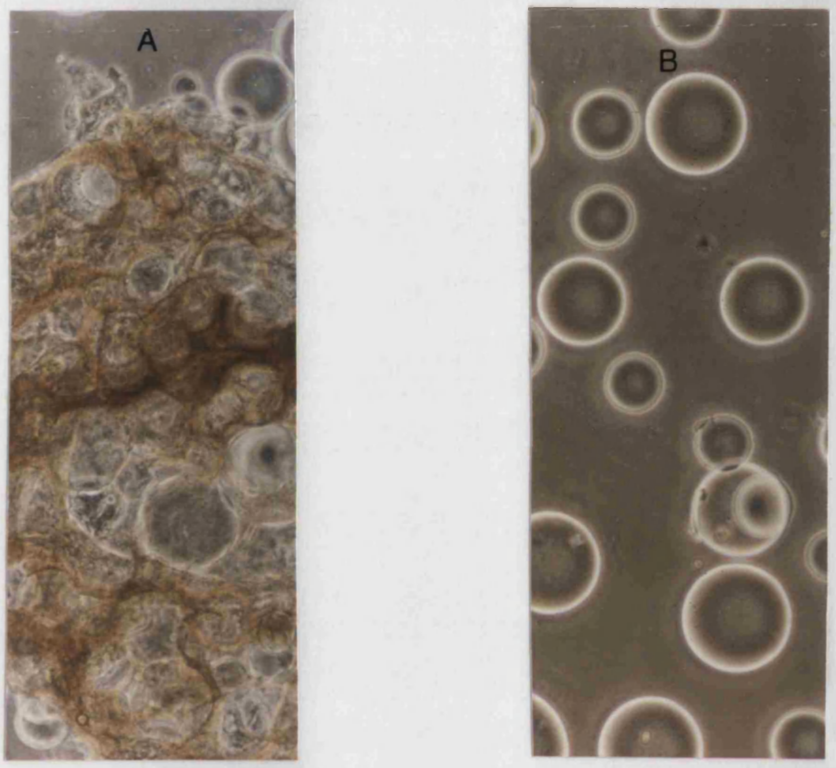


Figure 68. Aggregated Calmodulin Agarose Beads

It was found that the calmodulin agarose column formed clumps with continuous usage. On viewing the samples of the agarose contained in these clumps it was found that the agarose beads had aggregated together. Consequently a method was devised to avoid this happening. Figure A=aggregated calmodulin agarose beads. Figure B=non-aggregated calmodulin beads.

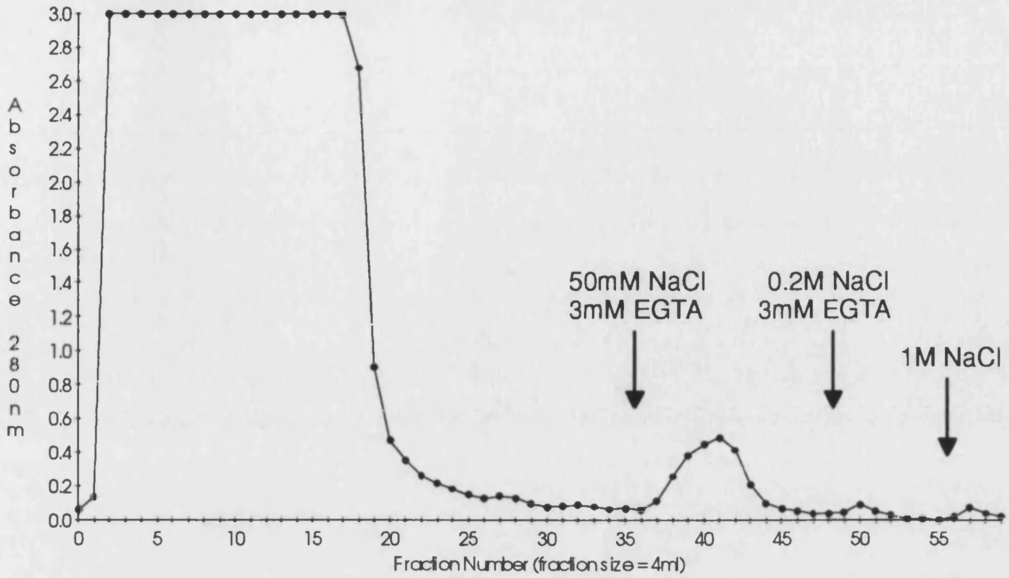
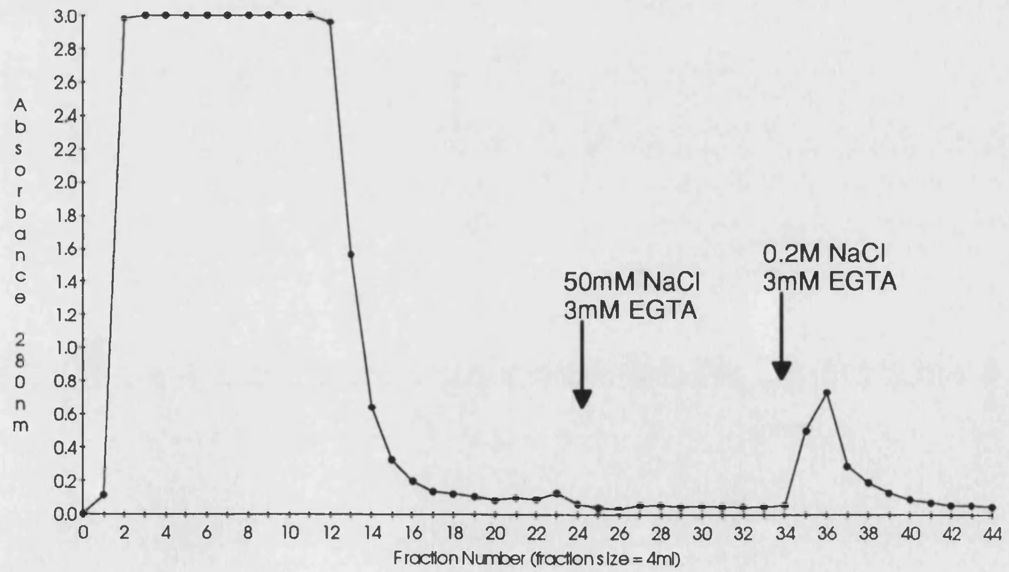


Figure 69. Elution Profile for New Calmodulin Agarose Isolating Calcium Dependent Calmodulin Binding Proteins

Due to the aggregation found in the previous batch of calmodulin agarose, the isolations were repeated using a new batch of calmodulin agarose.



`Figure 70. Elution Profile for Calcium Independent Calmodulin Binding Proteins Isolated with New Calmodulin Agarose

The calcium dependent calmodulin binding proteins were also isolated from rat testis so that a comparison between the two could be made. As it had become apparent that calcium dependent calmodulin binding proteins were eluted with 50mM NaCl and calcium independent ones with 200mM NaCl, the columns were washed first with buffer containing 50mM NaCl and then buffer containing 200mM NaCl. When the isolation was performed with the rat tissue being homogenised with calcium, two peaks eluted; whereas when the homogenising buffer contained EGTA only one peak resulted. This peak formed on the application of buffer containing 200mM NaCl.

The calmodulin binding proteins isolated from tapeworm and rat testes were analysed on a 12% polyacrylamide gel. See figure 71. Rat testis appeared to contain only one major calcium dependent calmodulin protein with a molecular weight of around 66kD in the first peak eluted with 50mM NaCl. The tapeworm calcium dependent calmodulin binding proteins obtained from the new agarose, consisted of 7 major bands with molecular weights of: 215, 116, 97, 66, 45 one between 45 and 36kD and one of around 24kD, whilst there appeared to be two major calcium-independent binding proteins, with molecular weights of 215kD and 45kD.

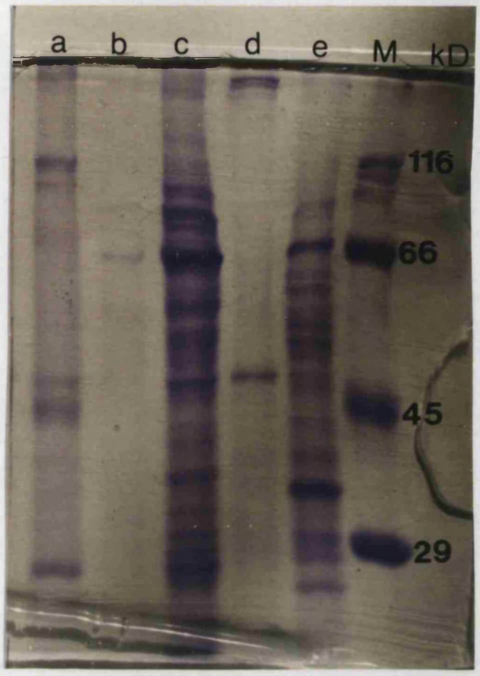


Figure 71. SDS-PAGE Analysis of Calmodulin Binding Proteins from New Agarose

This shows the calmodulin binding proteins isolated from both the tapeworm and the rat testis. Lane a=tapeworm calcium dependent calmodulin binding proteins, lane b=rat testes calcium dependent calmodulin binding proteins, lane c=rat application eluent with calcium, lane d=tapeworm calcium indepent calmodulin binding proteins and lane e=tapeworm application eluent with EGTA.

3.2.6 Immunocytochemistry

3.2.6.1 Preparation of Tissue

It was found that the tissue preservation was very poor when 4% (v/v) formaldehyde was used as the fixative. When viewed with the fluorescent microscope, the organelle structure was difficult to define, and the whole section had the appearance of being 'smeary'.

When 4% (v/v) gluteraldehyde was used as the fixative there was good tissue preservation with the organelles being well defined. However the plasma membranes do not stain very well. This was one reason for using the mixture of 5.5% (v/v) gluteraldehyde and 0.9% (v/v) osmium tetroxide as a fixative. With this mixture even better tissue preservation was obtained with very good contrast.

Unfortunately, there was a problem with segments from the middle and tail regions of the tapeworm, using any of the fixatives resulted in the resin blocks becoming brittle and prone to fragmenting on cutting. This made it exceedingly difficult to obtain thin sections for electron microscopy. One of the major reasons for this, was the presence of the oncospheres. As one moves down the tapeworm the proglottides contain progressively more and more oncospheres, with the terminal proglottides containing virtually nothing else. Consequently on sectioning the oncospheres fall out of the tissue leaving holes.

Another problem is that as one moves down the tapeworm the proglottids increase in size, for effective electron microscope processing tissue sections ought to be between 1mm-3mm^2. To preserve segments of this size would have meant that a proglottid would have to be cut in half, resulting in the loss of half of the proglottides contents.

Although this was tried, it failed as the resulting resin blocks disintegrated upon processing. Consequently, whole proglottids were used, with the result that the tissue was improperly infiltrated with resin, so having a 'lacy' like appearance. An example of this can be seen in figure 72.

There are ways in which the infiltration problem could have been overcome. One would have been to leave the tissue in fixative for longer than one hour, ideally a minimum of 24 hours. However, this would almost certainly have resulted in either the loss of the antigen of interest, or extensive cross-linking with gluteraldehyde that would result in unrecognisable epitopes on the antigen. Likewise the tissue could have been left for longer in the resin, or a different resin used. LR-White was used because it will polymerise at low temperatures in this case 55°C, which minimises the denaturation of the proteins. Higher temperatures for polymerization required by many other resins' results in a less viscous resin that is able to permeate the tissue more readily. However, this would result in heat denaturation of the antigens. Sometimes infiltration problems can be overcome by performing the initial stages of infiltration under vacuum, for brief periods. This was tried with the tapeworm segments, and better infiltration was observed. But, when the sections were viewed under the electron microscope much of the internal tissue structure had been rearranged, and isolated organelles were present within the resin but not related to any other tissue structures. Consequently these could not be used for immunocytochemical work.

The treatment of some of the blocks with 1% (w/v) tannic acid was used to enhance the contrast of the membranes within the tissues, in particular the neural tissue which

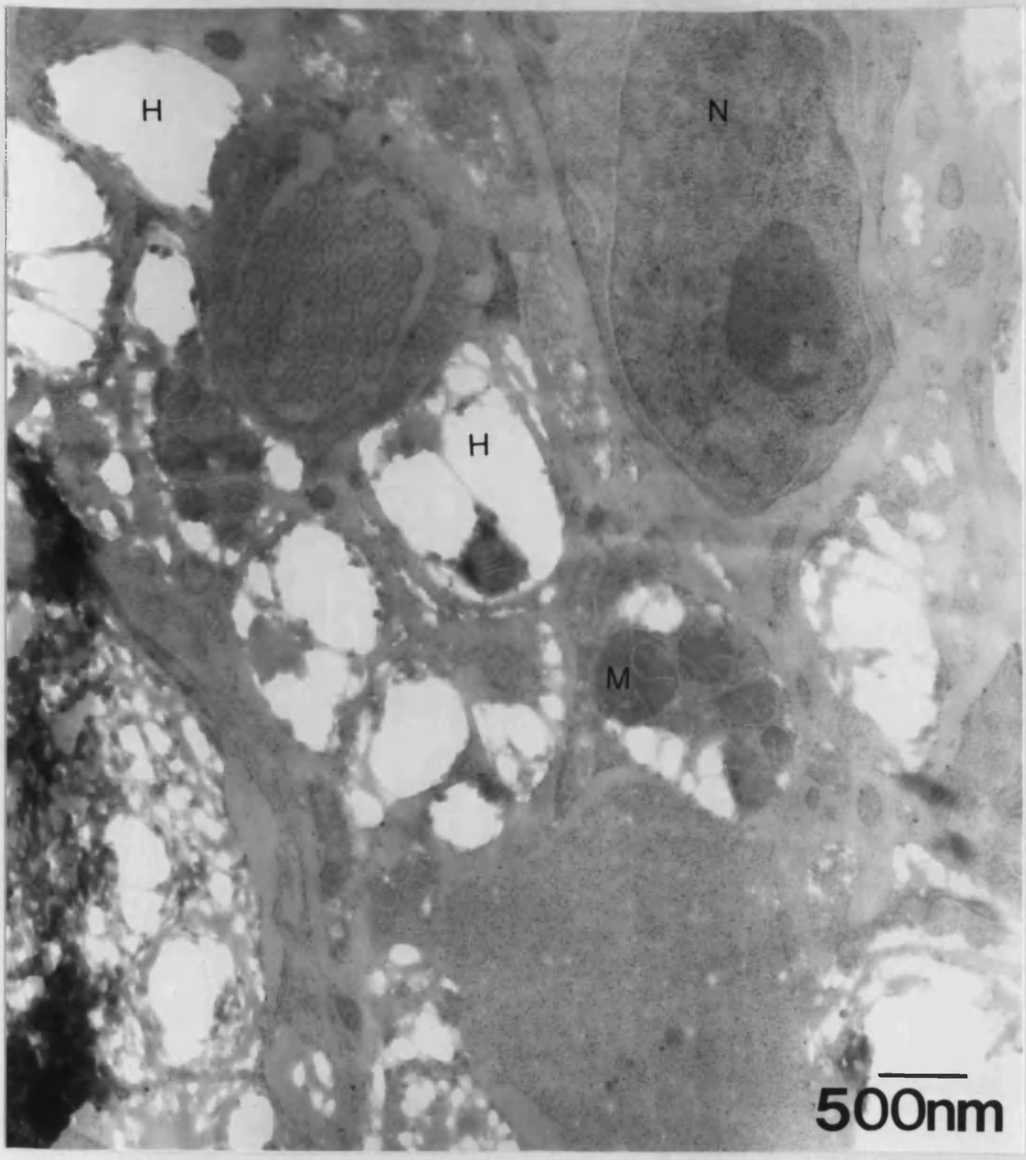


Figure 72. Electron Micrograph Showing the Infiltration Problem for the Cestode Material

The tapeworm tissue was found to have not been fully infiltrated by the resin, resulting in a lacy like appearance of sections when viewed with the electron microscope. H=hole, M=mitochondria, N=nucleus, F=flame cell. x12k.

is notoriously difficult to define in tapeworm tissue (Webb & Davey 1975). On sections from the upper regions of the tapeworm, this did enhance the contrast and definition of the tissue. However, in lower regions it only served to increase the crumbliness of the resin blocks.

Another problem that was encountered was the pre-infiltration with a mixture of 2:1 LR White:70% (v/v) ethanol. This solution was found to go cloudy on the addition of the tapeworm segments. The reason for this is that, once LR-White is prepared into solution it begins to slowly polymerize. The result is a decreased tolerance to any water that may still be in the tissue samples. This factor may also have contributed to the difficulties encountered with infiltration. However, the LR-White being used initially was near the end of its shelf life (three months), being two months old. So a fresh batch was purchased and the problem of clouding diminished.

One other difficulty was found in the polymerized blocks, where the resin appeared to have hardened to different degrees. LR-White is supposed to be a 'soft' resin however it was found that within one block the resin could vary from being 'rock' hard to 'soft'. Where the blocks were 'rock' hard, a diamond knife had to be used to obtain thin sections. This did create a problem, though, whilst one was trimming the blocks in preparation for thin sectioning because the blocks would fracture along the lines between the 'rock' hard resin and the 'soft' resin.

Despite all these problems thin sections were obtained for each region and could be used for immunocytochemical staining.

3.2.6.2 Immunofluorescence.

The immunofluorescent studies worked satisfactorily, however the fluor used was FITC that has a green/yellow colour. Unfortunately, it was found that certain regions of the tapeworm auto-fluoresced, with a yellow colour, in particular the oncospheres and the tegument. But, one could still easily distinguish where the fluor had selected. The autofluorescence was reduced considerably by pretreating the sections with acetone.

It is very difficult to obtain a satisfactory permanent record of the results obtained. Figures 73-76 show four examples of the results. The fluorescent label was found associated with the parenchyma, muscle blocks, within the oncospheres, and in what appeared to be channels in the tegument. The results were encouraging enough for the study to be undertaken at the electron microscope level, where more information concerning the location of calmodulin could be discerned.

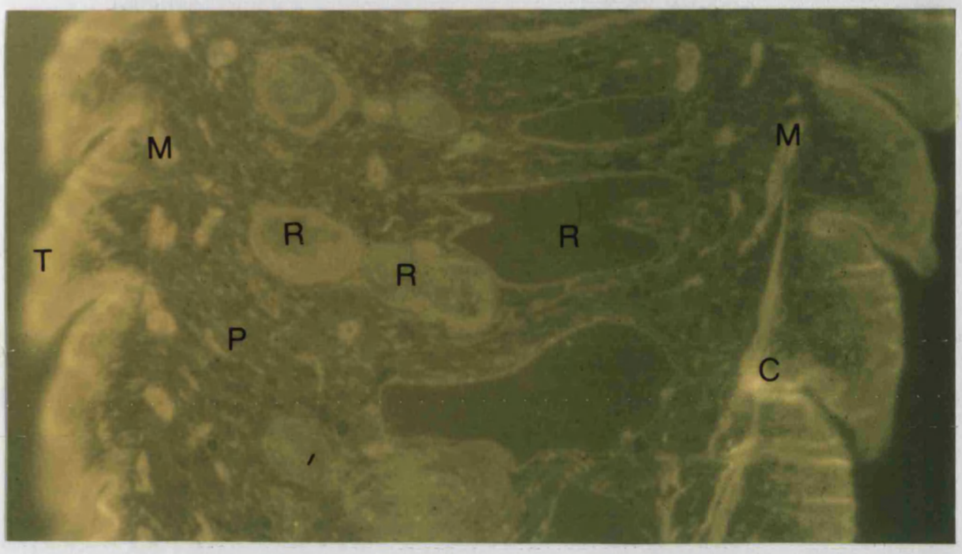


Figure 73. Photograph of Fluorescent Labelled Section of the Tapeworm at Low Power

This shows a section of the worm consisting of several proglottides. P=parenchyma, T=tegument, M=muscle, R=reproductive organs, C=brightly stained 'canals' which run from the exterior of the tegument to the interior of the worm.

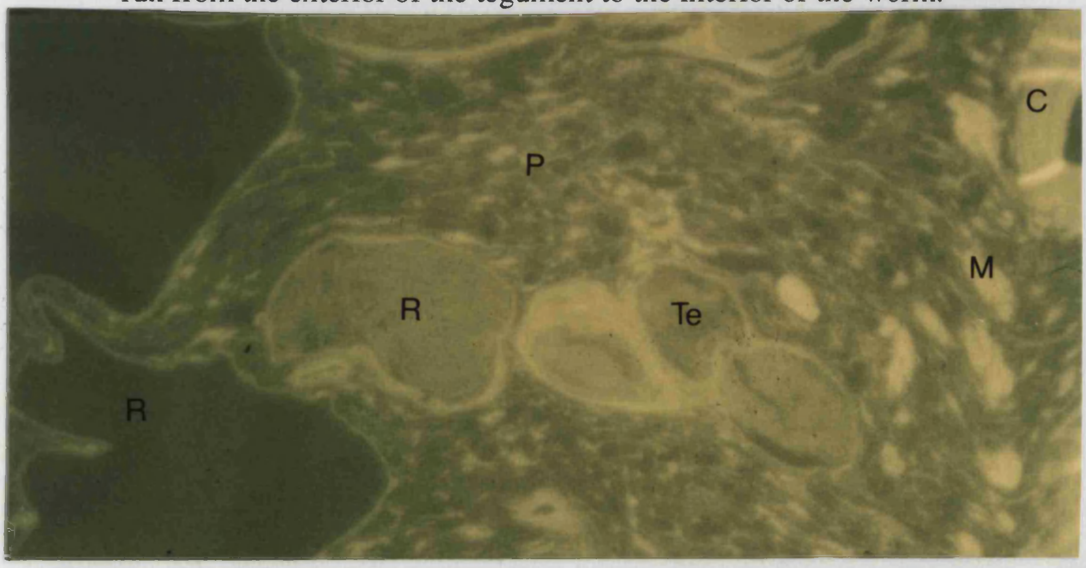


Figure 74. Higher Magnification of the Reproductive Organs

Te=testes, R=reproductive organ, P=parenchyma, M=circular muscle, C='canals'.

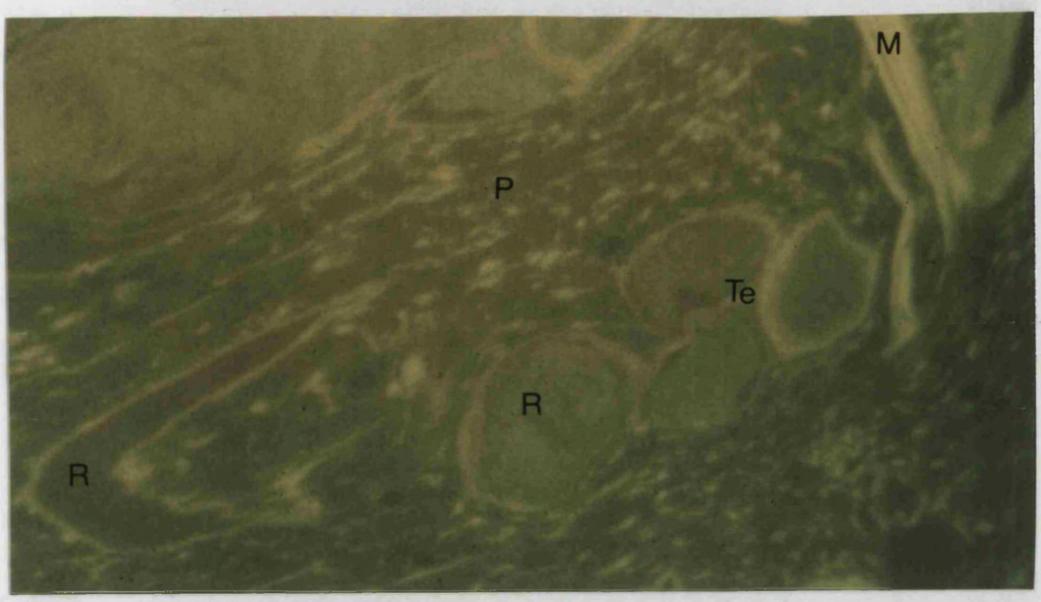


Figure 75. High Magnification of the Reproductive Organs

Here fluorescent label is seen with the edges of the reproductive organs, the longitudinal muscle and throughout the parenchyma. R=reproductive organ, Te=testes, P=parenchyma, M=longitudinal muscle.

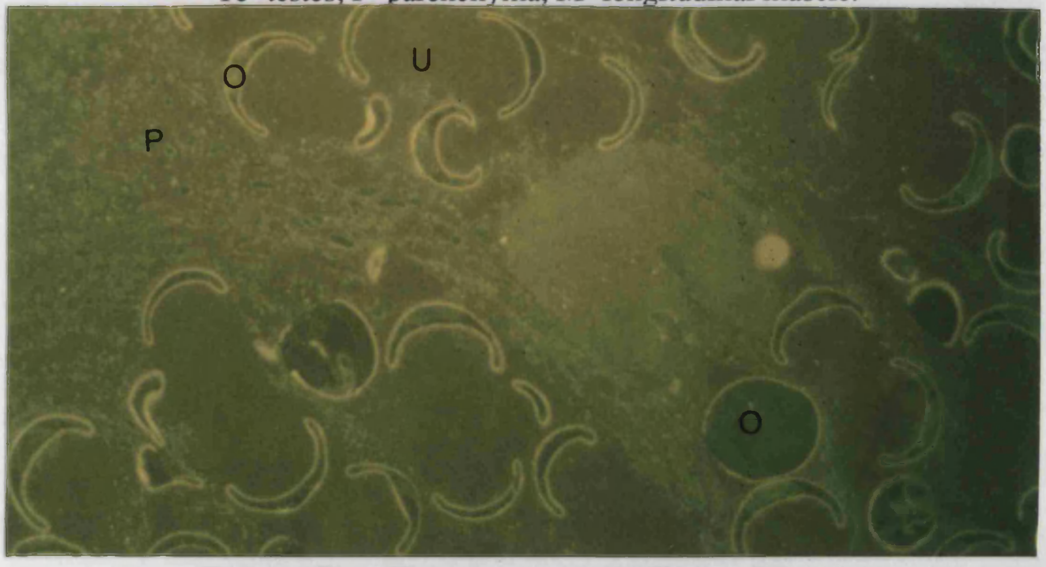


Figure 76. Fluorescent Labelling of Uterus

Fluorescent label can be seen throughout the parenchyma and within some oncospheres. P=parenchyma, U=uterus, O=oncosphere. Note that the outer shell of the oncospheres appears yellow which is due to natural autofluorescence.

3.2.6.3 Immunogold Labelling

Initially thin sections were collected on copper grids. However, it was found that other workers recommend using either nickel or gold grids. Consequently, all subsequent sections were collected on nickel grids (Beesley 1989).

The anti-calmodulin antibody used for the immunocytochemistry was checked for its recognition of calmodulin by performing an ELISA with commercial calmodulin. It was also later used for western blots of protein isolates. See section 3.2.4.

The controls using the antibody pre-sorbed with calmodulin were completely free from gold labelling. As the same set of antibodies were being used throughout, this control was only performed once, in triplicate, for each tissue processing method and for each region of the tapeworm, due to the limited supply of calmodulin and antibodies.

The control used routinely in all experiments was the treatment of samples with just the second gold-conjugated antibody. Generally these controls were free of gold-labelling. However, if fresh blocking solutions (bovine serum albumin, glycine or gelatine) were not used then a small background labelling was seen. This usually consisted of between one and ten gold labels across the entire thin section. These non-specifically bound labels, were always on there own and never formed groups although, they did tend to be found on the very outer edges of nuclei.

Consequently, gold particles detected on the sections represents binding of the antibody to either calmodulin or calmodulin associated with one of its target proteins, or a protein where calmodulin forms a permanent regulatory subunit.

In all sections of the worm, the 'head', neck, upper middle, middle and tail the calmodulin label was found associated with flame cells, cytons and muscle fibres.

Figure 77 shows antibody label associated with a flame cell, part of the excretory system (section 1.2.1.2.) from the head region. Figures 78 to 84 show calmodulin localised to the cytoplasm of the cytons, the nuclear membrane, the nucleus associated with some chromatin, and within the nucleolus. One of the most abundantly labelled structures throughout the tapeworm tissue was the muscle fibres See figures 85 to 88. Figure 87 is taken from the neck region and besides showing calmodulin associated with many muscle fibres shows an organelle composed of electron dense granules. Several of these structures were detected in the neck region and in each case they were always packed with gold label. This figure also shows calmodulin present in the cytoplasm and associated with mitochondria.

Calmodulin was also found in the microthrix and the distal cytoplasm, as can be seen in figures 89 to 92. Below the distal cytoplasm is the basal lamella through which any substances for secretion through the tegument have to pass. To aid this passage of substances to the distal cytoplasm are internuncial processes (section 1.2.1.1., figure 14.). Calmodulin label was frequently observed in the cytoplasm surrounding these processes. Figure 93.

Within the reproductive organs calmodulin was found associated with spermatozoa. See figures 94 and 95. However, it was difficult to asses with certainty which organ they were associated with as once produced in the testes' spermatozoa migrate to the seminal receptacle passing through the external and internal seminal vesicles (see section 1.2.1.2. and figure 14). No calmodulin was found associated with the ovaries. Figure 96 shows what is thought to be part of an oncosphere. Identification of oncospheres at such high magnification was difficult due to the problems with infiltration.

In the tail region of the cestode, there were a large number of organs/organelles which contained electron lucent granules. Frequently calmodulin was found associated with these granules. See figure 97.

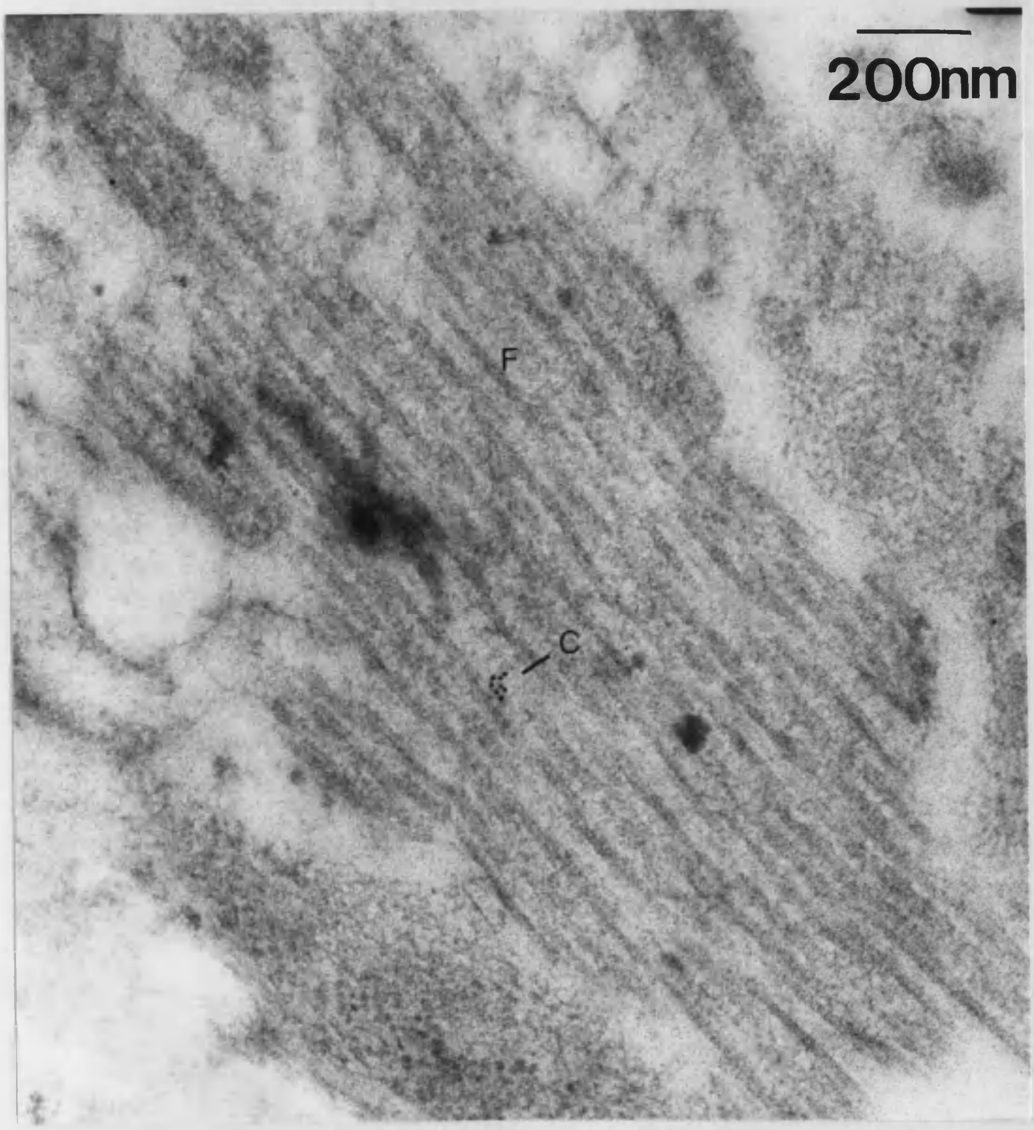


Figure 77. Electron Micrograph of a Flame Cell

Calmodulin label was frequently found associated with flame cells which form part of the excretory system in cestodes. This section was from the head region and was fixed with gluteraldehyde. F= Flame Cell, C= calmodulin. x40k.

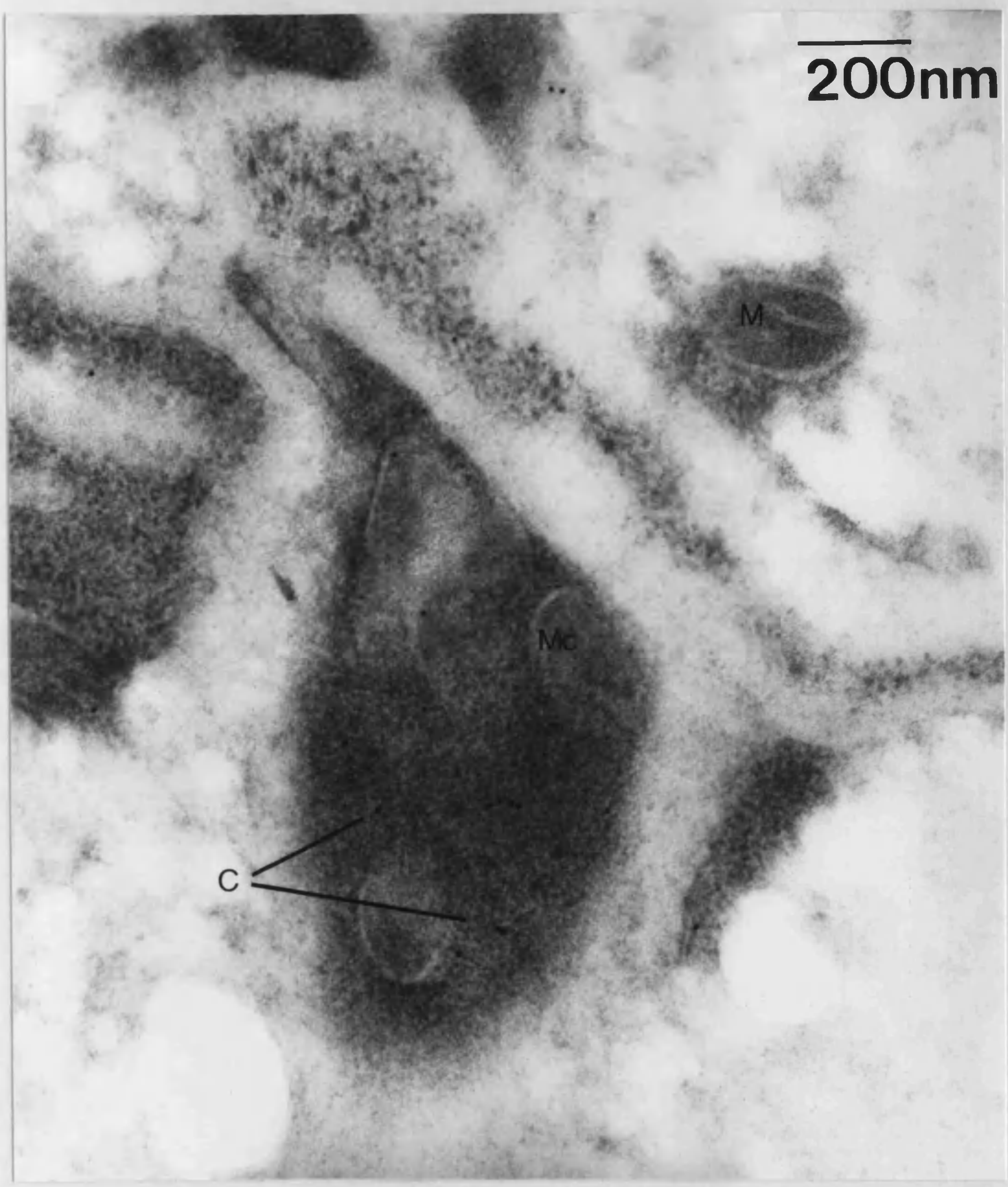


Figure 78. Cross Section of the Cytoplasm of a Cyton

This is a section treated with gluteraldehyde taken from the head region. The cytoplasm of a cyton can be seen containing calmodulin. The granular appearance of the cytoplasm is due to the ribosomes. C= calmodulin, Mc= myocyton, M= mitochondria. x40k.

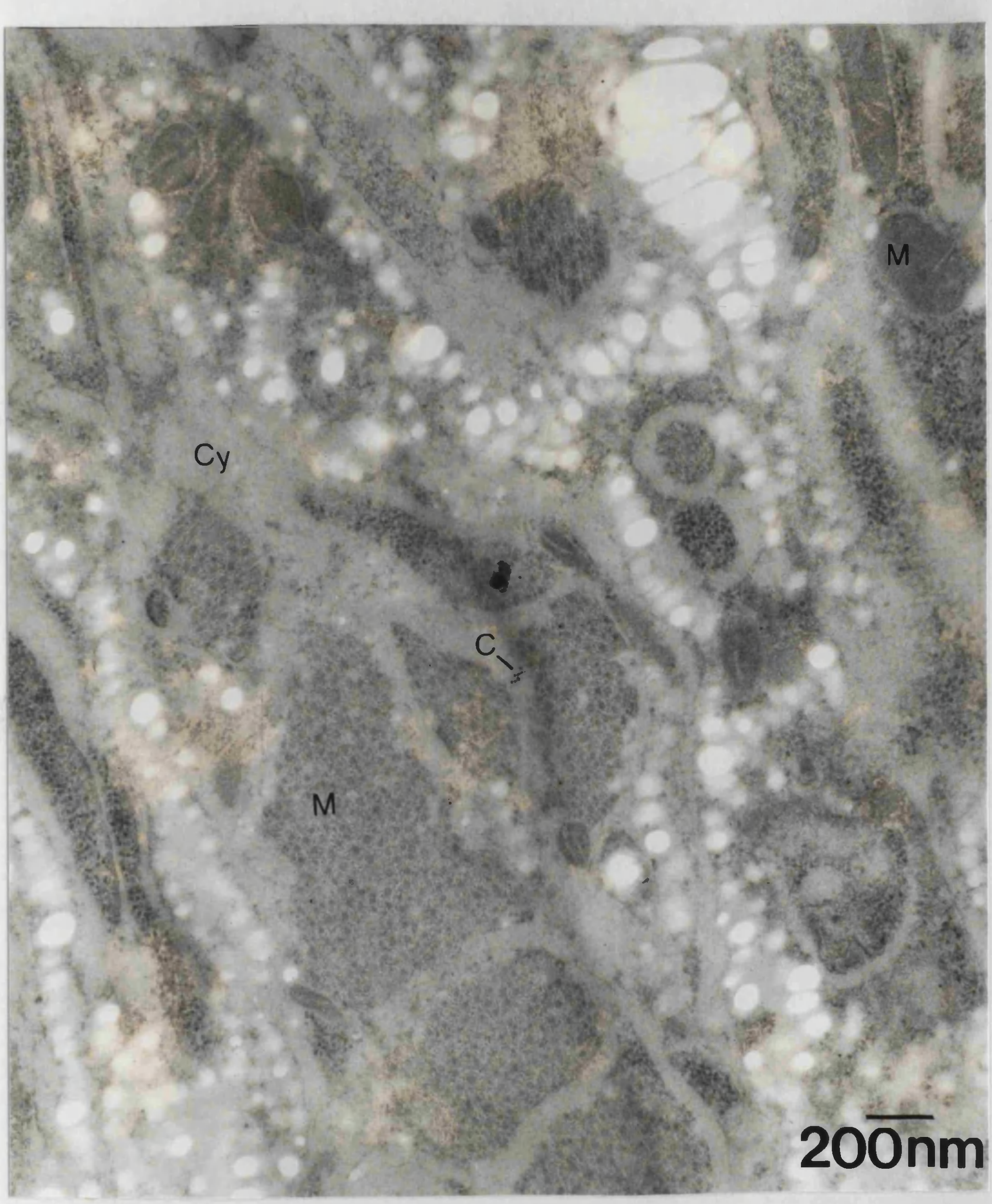


Figure 79. A Cross-Section Through Myofibrils

Calmodulin label can be seen in the cytoplasm between two muscle blocks, on this section taken from the upper middle region of the tapeworm. The tissue has been fixed with gluteraldehyde. C= calmodulin, Mf=myofiril, Cy=cytoplasm, M= mitochondria. x20k.

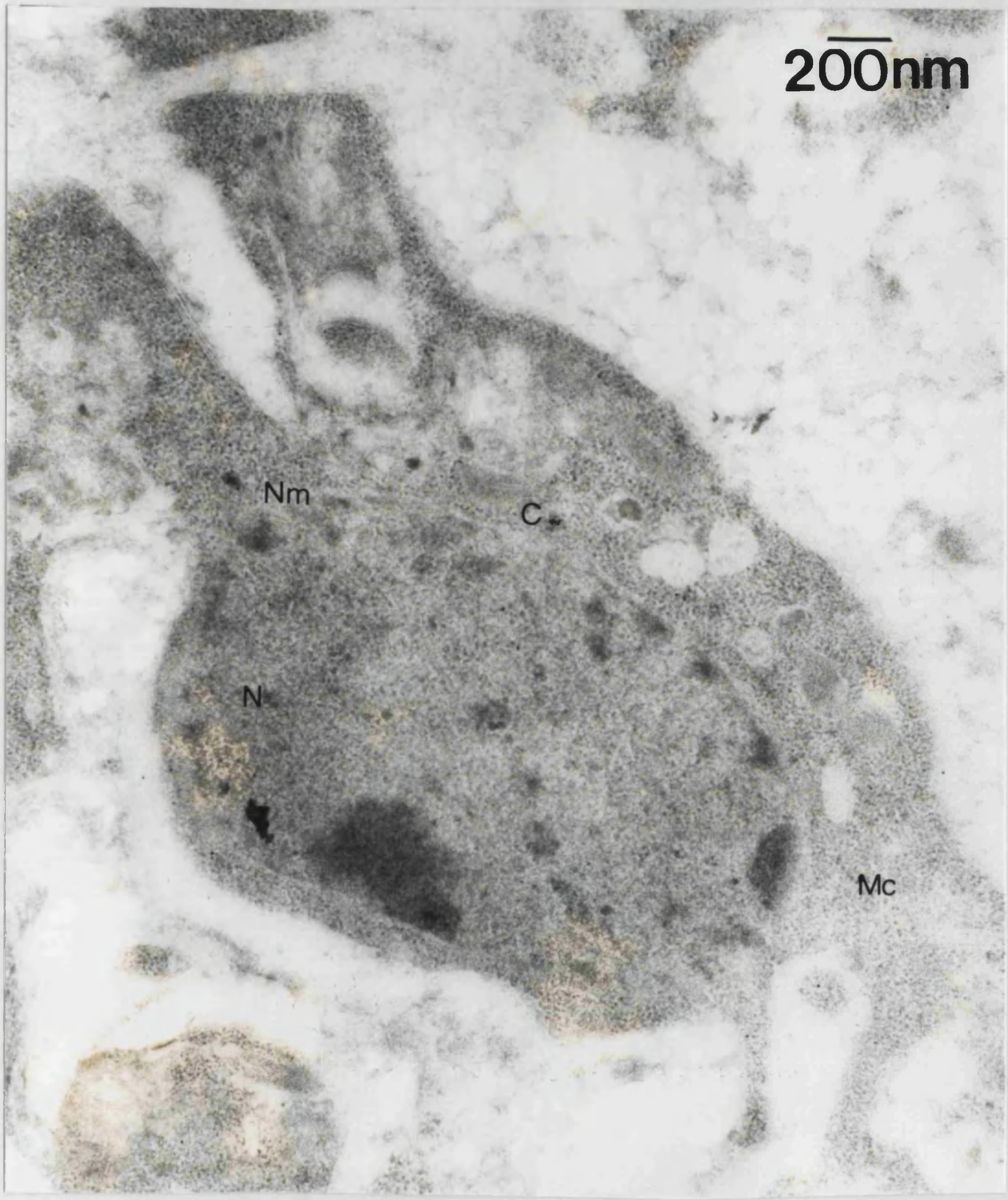


Figure 80. Nucleus of a Cyton

A group of calmodulin antibodies can be seen in the cytoplasm adjacent to the nuclear membrane in this section from the head region which was fixed with gluteraldehyde. N=nucleus, Nm = nuclear membrane, C= calmodulin and Mc = myocyton. x20k.

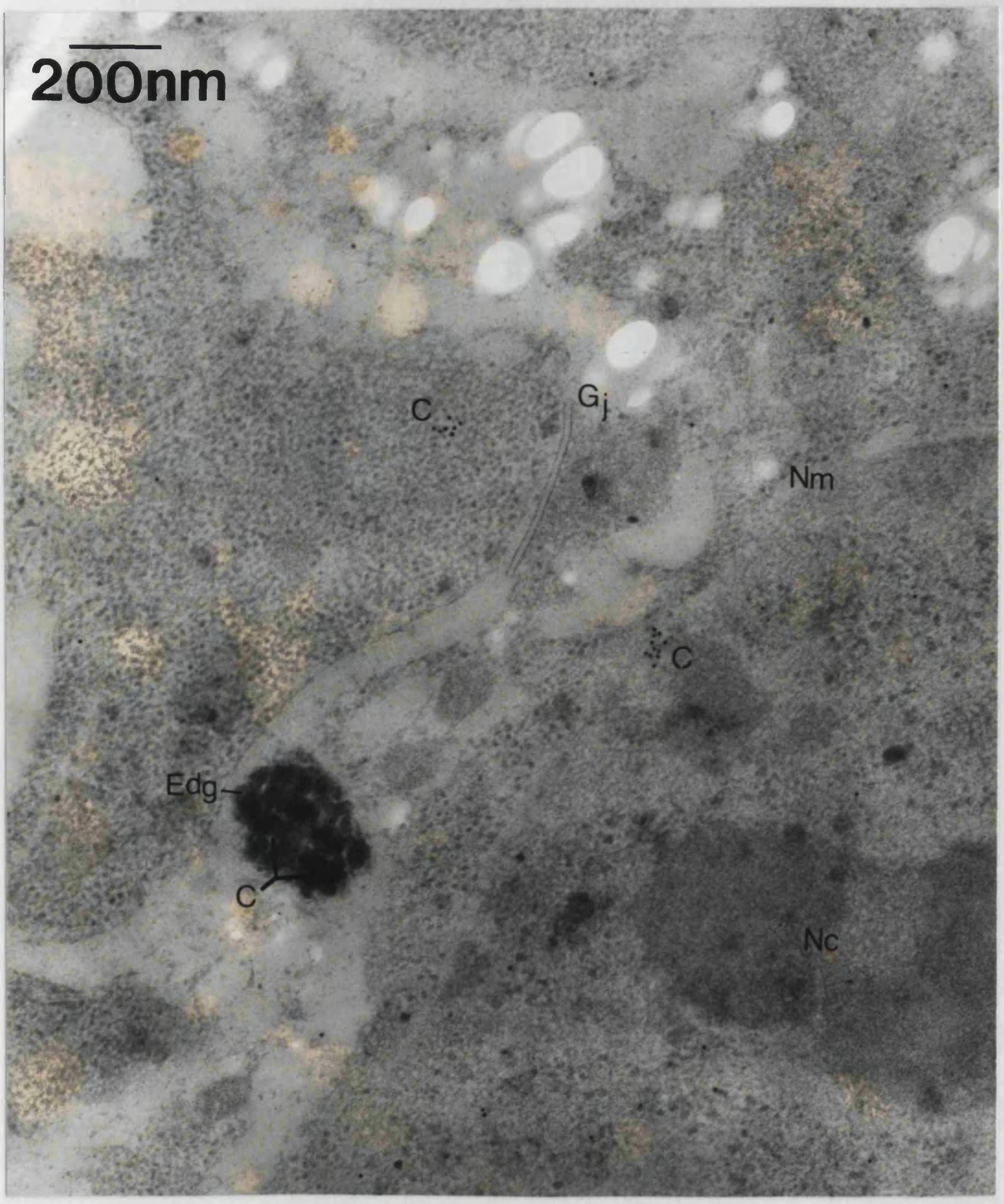


Figure 81. Gap Junction of Two Cytons

This figure shows the junction between two cytons, forming a gap junction in the neck region of the tapeworm. The tissue was fixed with gluteraldehyde. Calmodulin can be seen in the cytoplasm, associated with the nuclear membrane and associated with an unidentified organ which could be neurosecretory. C=calmodulin, Nc=nucleolus, Nm=nuclear membrane, Gj=gap junction, and Edg=electron dense granules. x30k.

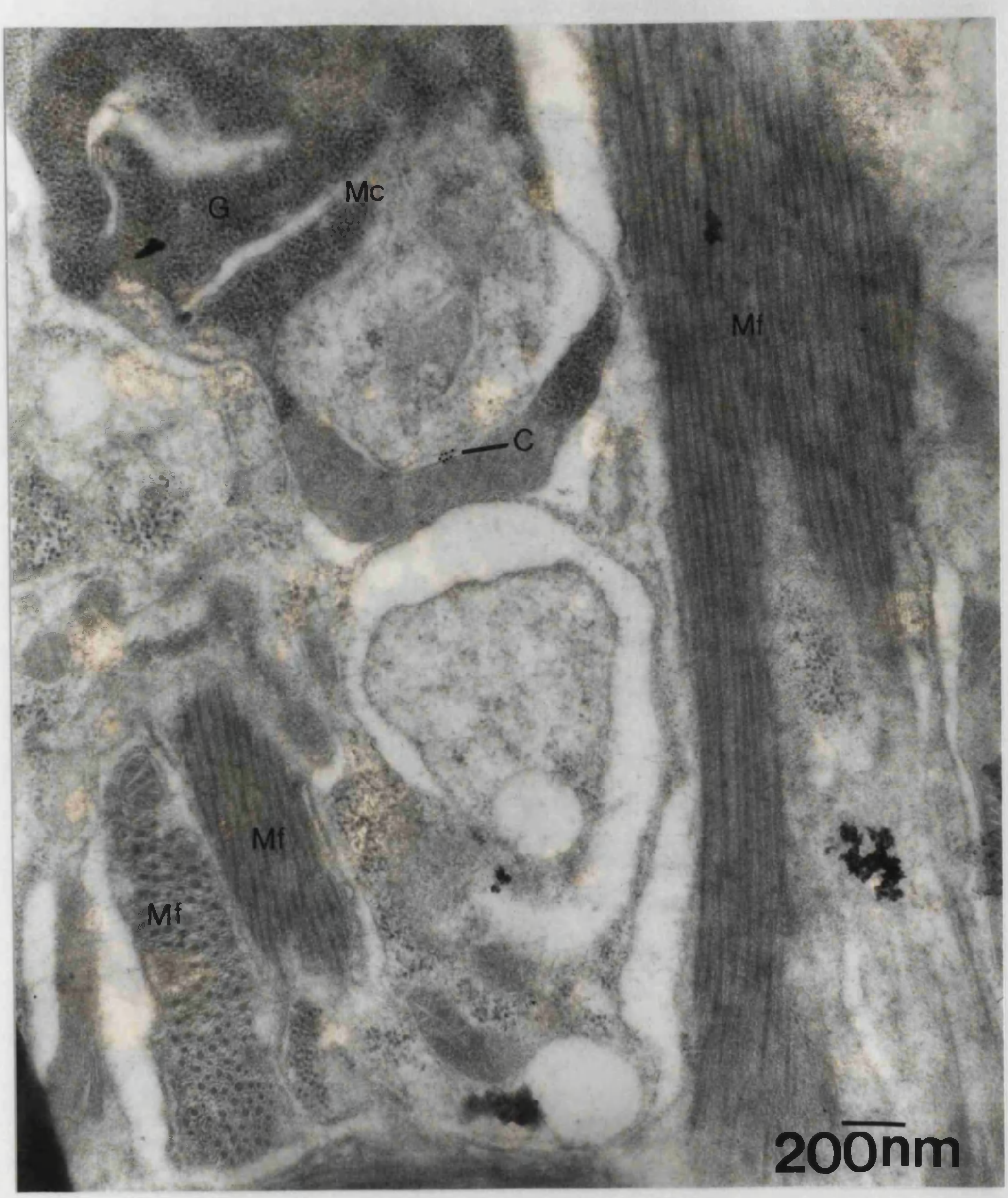


Figure 82. Myofibrils and Myocytons

This section, fixed with gluteraldehyde and osmium tetroxide and treated with tannic acid, shows calmodulin associated with a plasma membrane which is probably a nuclear membrane. Glycogen granules are visible in the myocyton which provide the energy for the myofibres they serve. This section was taken from the head region. Mf=myofibres, Mc=myocyton, G=glycogen, and C=calmodulin. x20k.

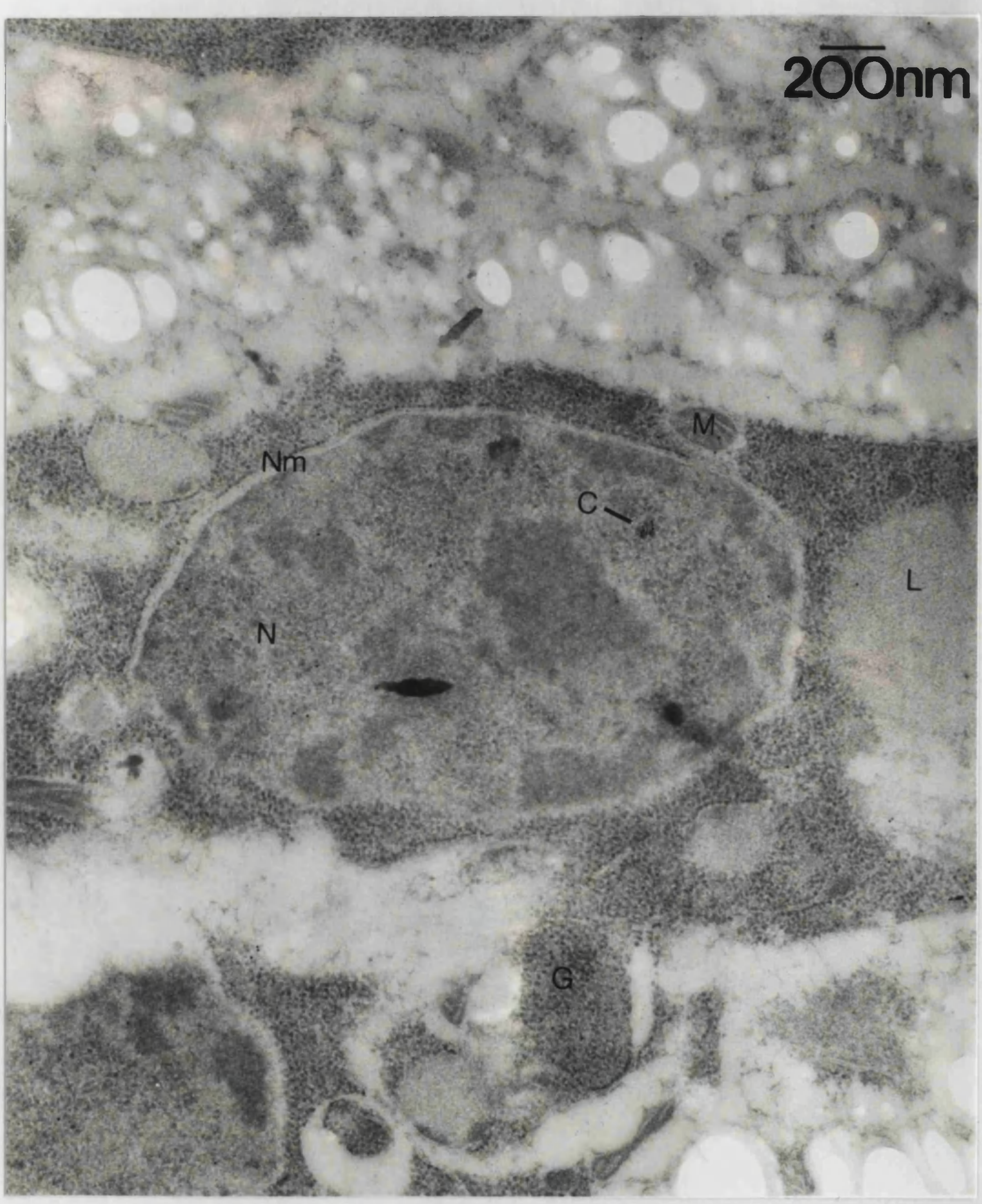


Figure 83. Nucleus of a Cyton

This shows calmodulin within the nucleus from a section from the neck region fixed with gluteraldehyde. Chromatin is present forming irregularly shaped 'clumps'. N=nucleus, Nm=nuclear membrane, C=calmodulin, M=mitochondrion, L=lipid and G=glycogen. x20k.

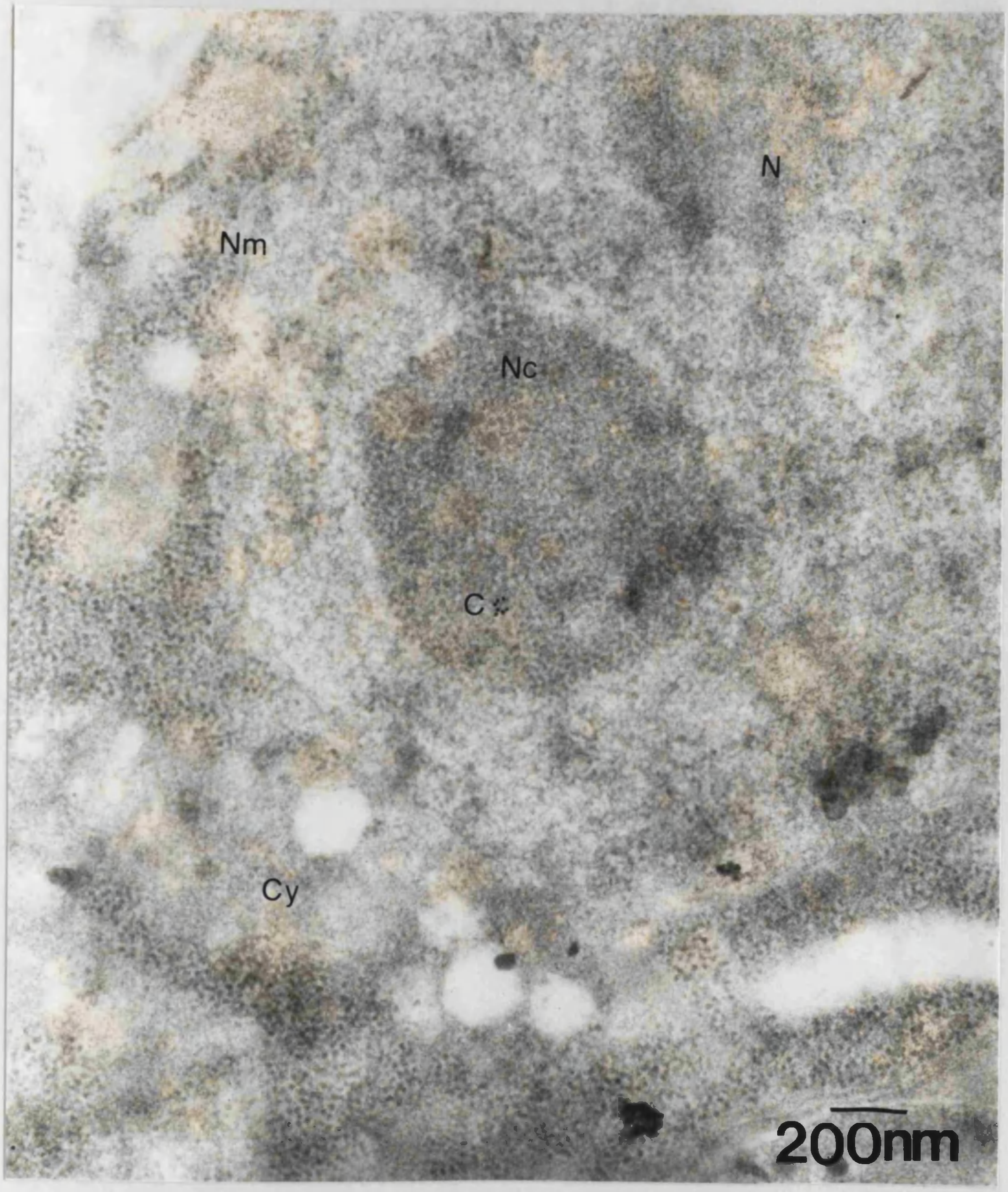


Figure 84. A Nucleolus

Calmodulin label can be clearly seen in the nucleolus of this nucleus from the head region. The tissue was fixed with gluteraldehyde and osmium tetroxide and treated with tannic acid. N=nucleus, Nm=nuclear membrane, Nc=nucleolus, C=calmodulin and Cy=cytoplasm. x30k.

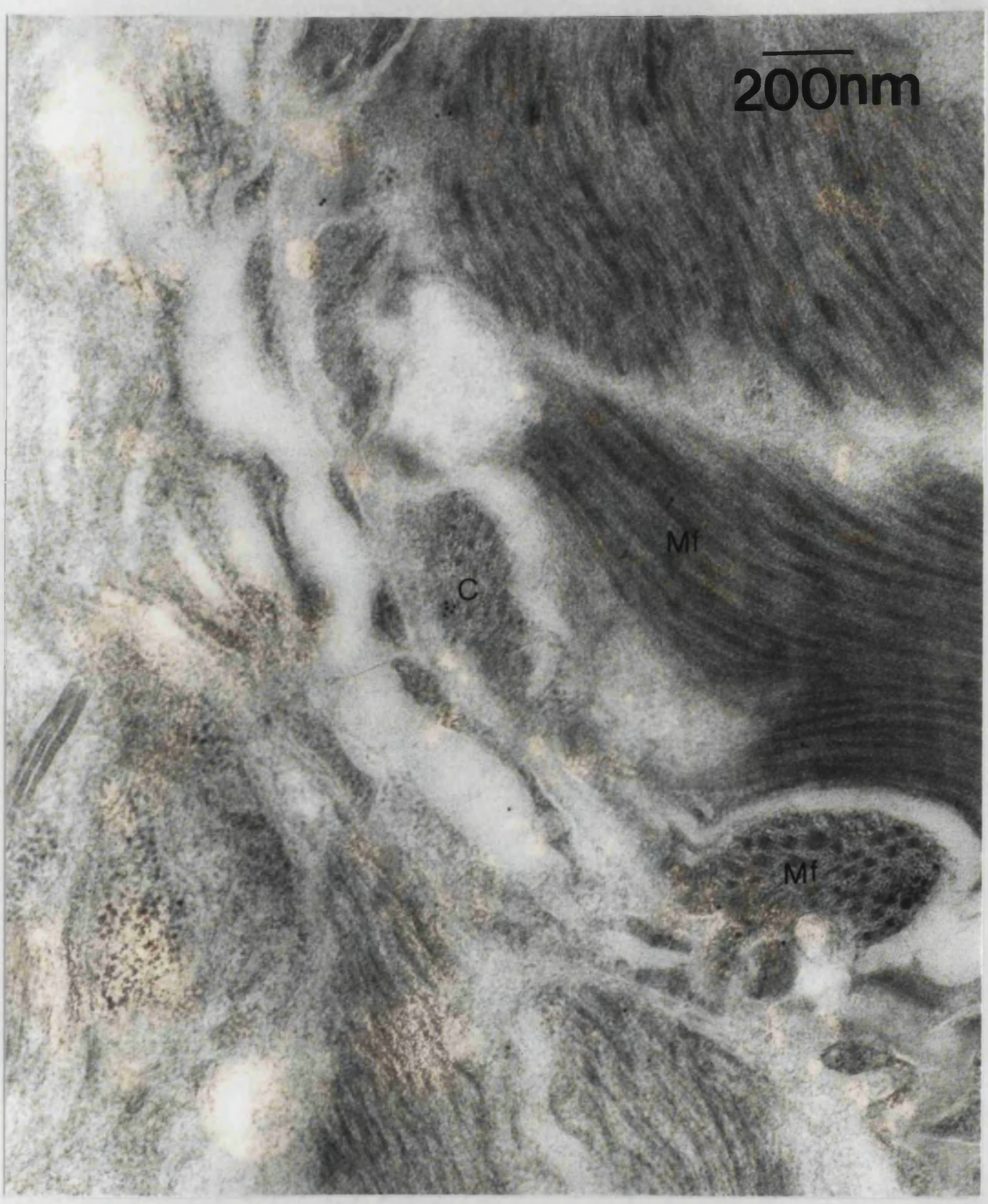


Figure 85. Myofibres

This shows a section from the head region with the tissue having been fixed with gluteraldehyde and osmium tetroxide. Calmodulin is associated with myofibrils which form the muscle tissue. Mf=myofibrils and C=calmodulin. x30k.

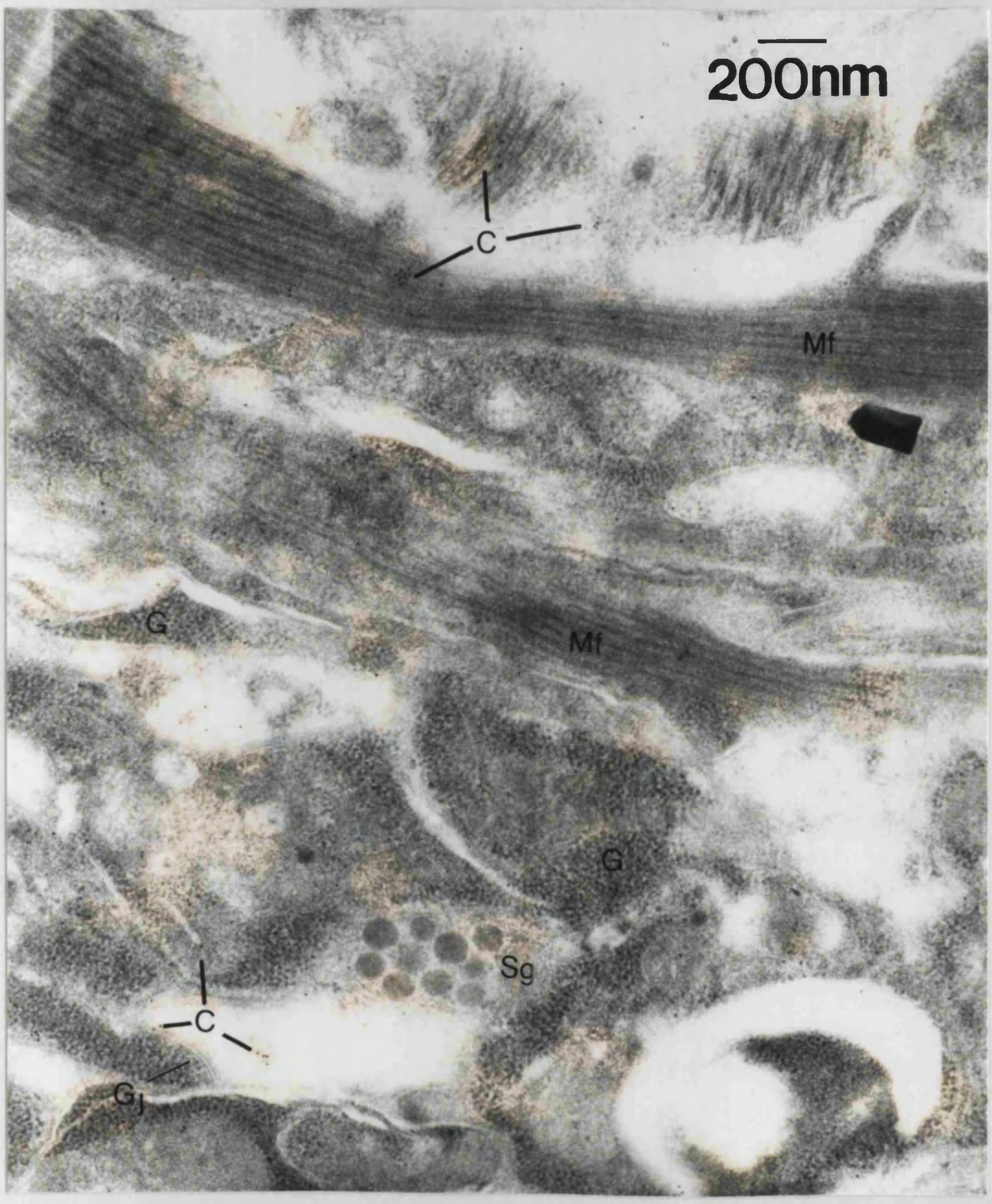


Figure 86. Myofibrils and Cytons

In this section from the head region calmodulin can be seen associated with myofibrils and in the cytoplasm of the cytons. The tissue was fixed with gluteraldehyde and osmium and then treated with tannic acid. Mf=myofibrils, C=calmodulin, Sg=secretory granules, and G=glycogen. x20k

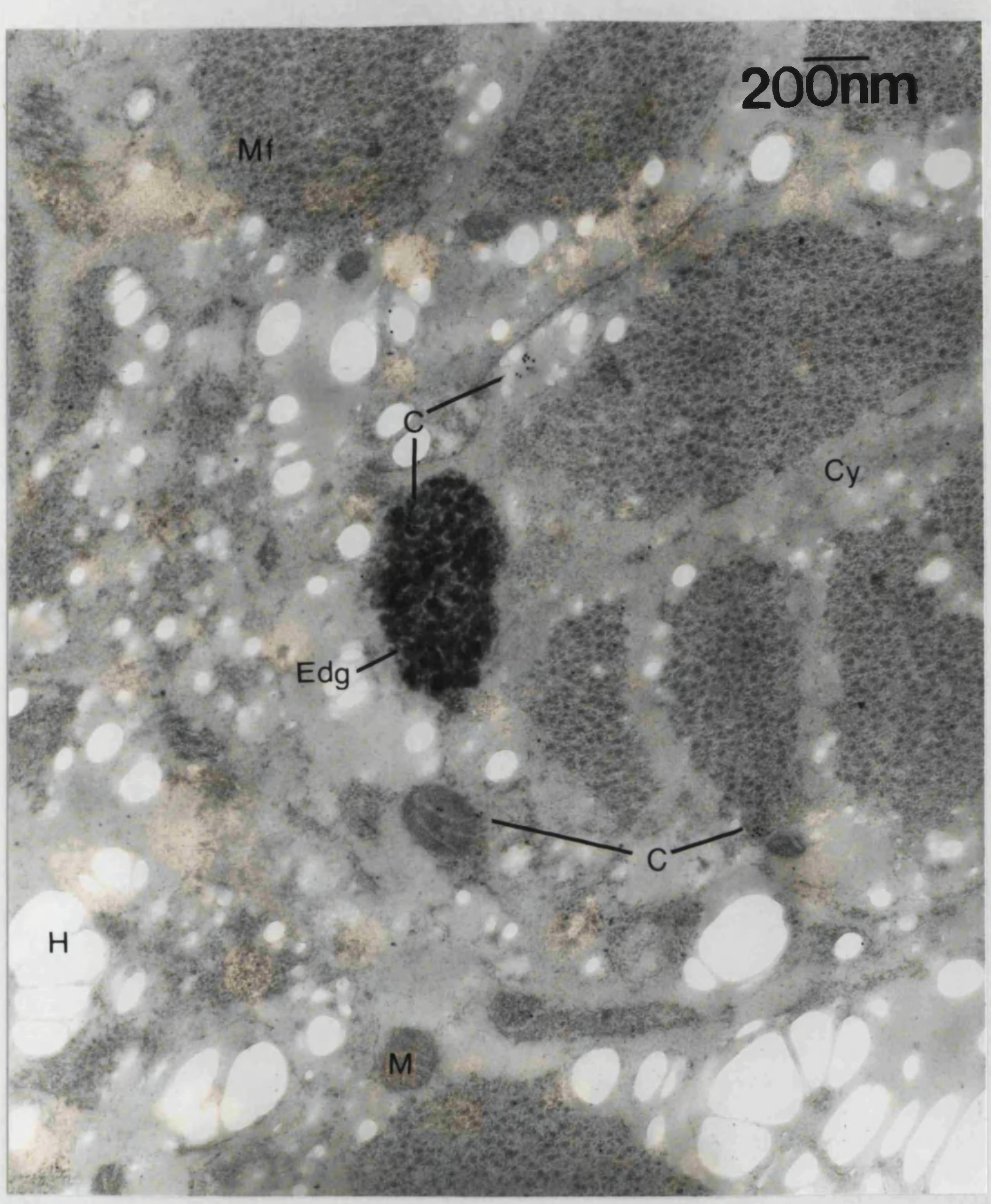


Figure 87. A Region of Muscle Tissue

This shows several myocytons converging around an organelle which appears to be composed of electron dense neurosecretory granules. Calmodulin is localised to the cytoplasm, the myofibrils and to the electron dense granules. This is taken from the neck region and was fixed with gluteraldehyde. Mf=myofibril, C=calmodulin, Edg=electron dense granules, M=mitochondria and Cy=cytoplasm. x20k.

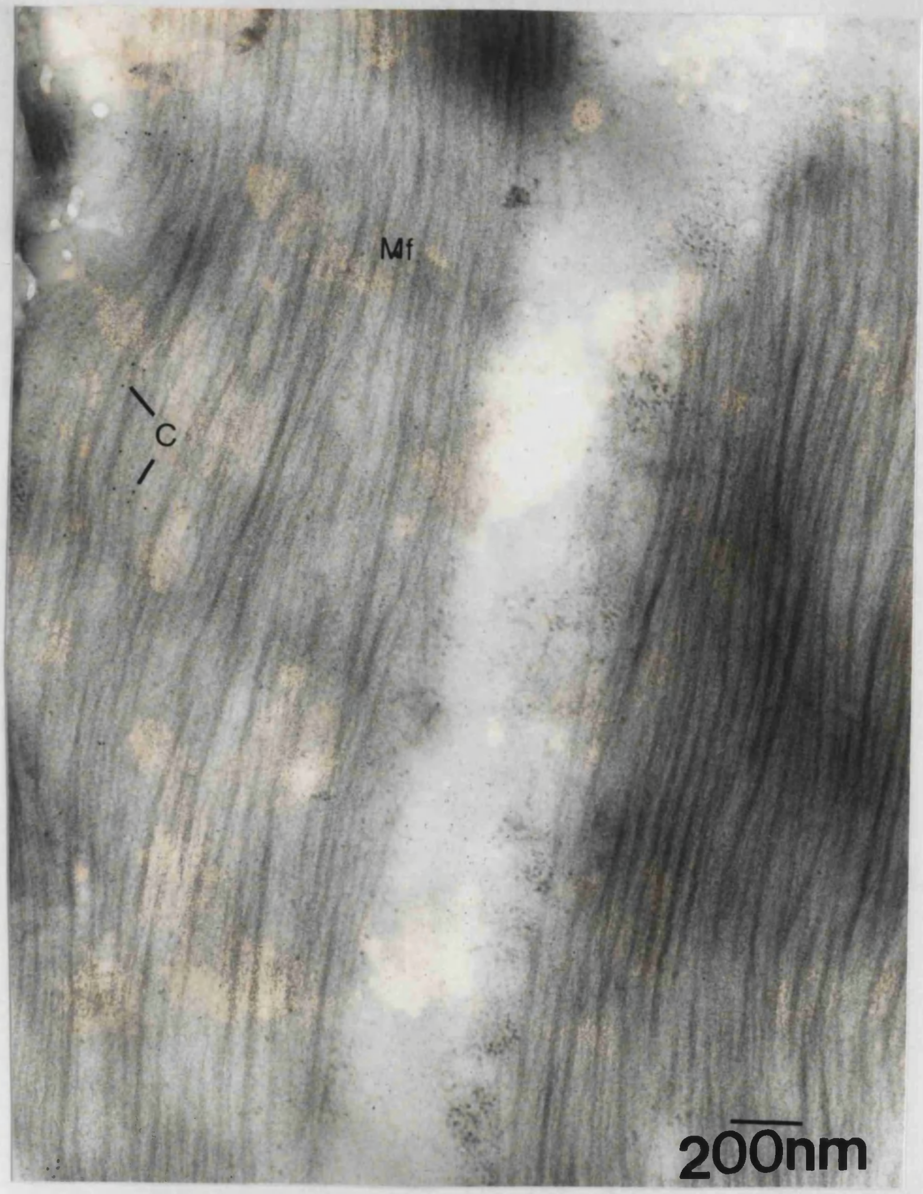


Figure 88. Muscle Fibres From The Tail Region.

Calmodulin is seen associated with the myofibres of this section from the tail region.

The tissue was fixed with gluteraldehyde. Mf=myofibril and C=calmodulin.x20k.

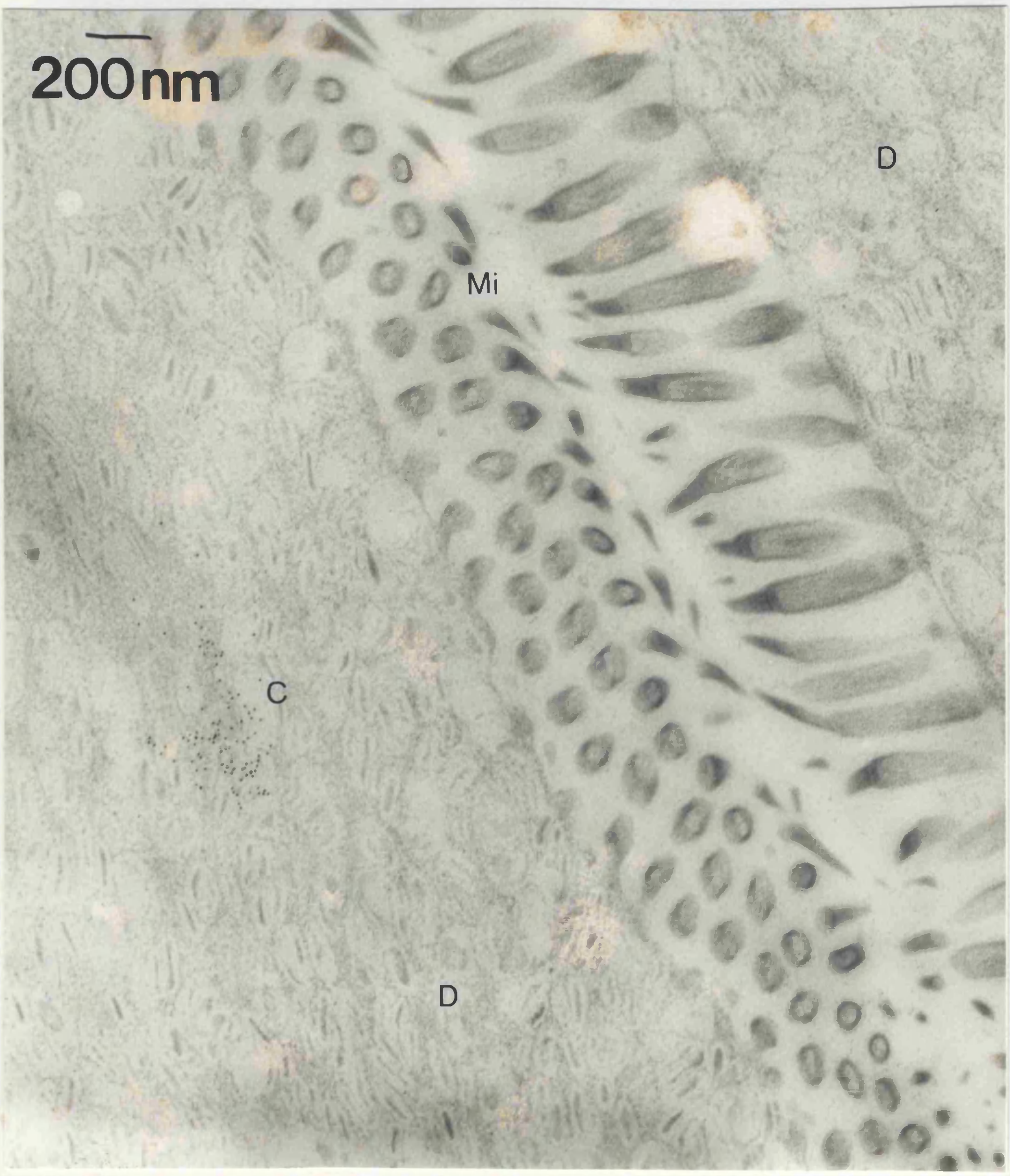


Figure 89. Tegument From The Tail Region

This is a section from the tail region showing the distal cytoplasm and the microthrix. The tissue was fixed with gluteraldehyde. Mi=microthrix, C=calmodulin and D=distal cytoplasm. x20k.

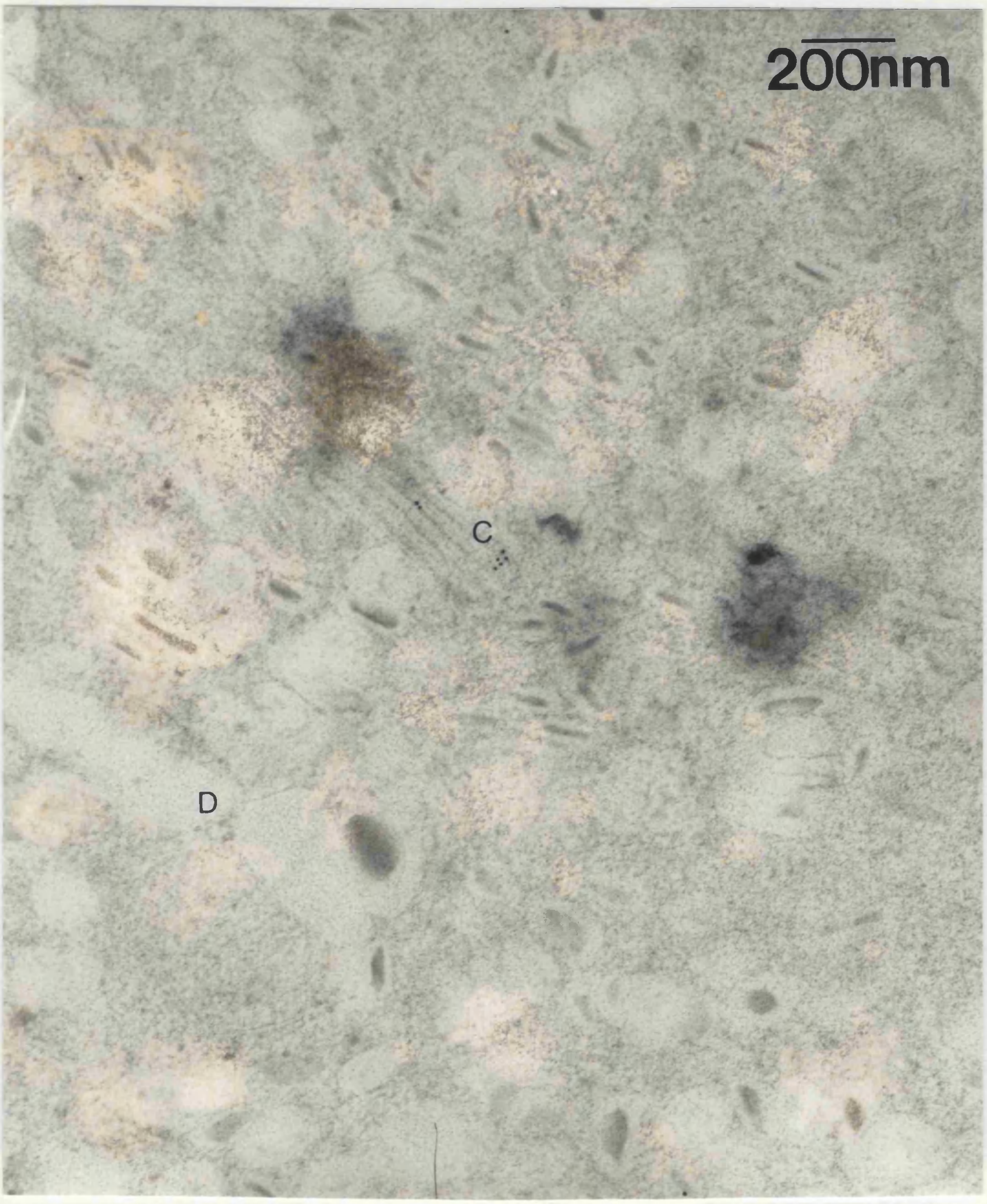


Figure 90. A Discoid Granule in the Distal Cytoplasm

The distal cytoplasm is composed of a large number of discoid granules, to which calmodulin label was frequently associated. This shows a section for the head region where the tissue was fixed with gluteraldehyde. D=distal cytoplasm, C=calmodulin. x30k.

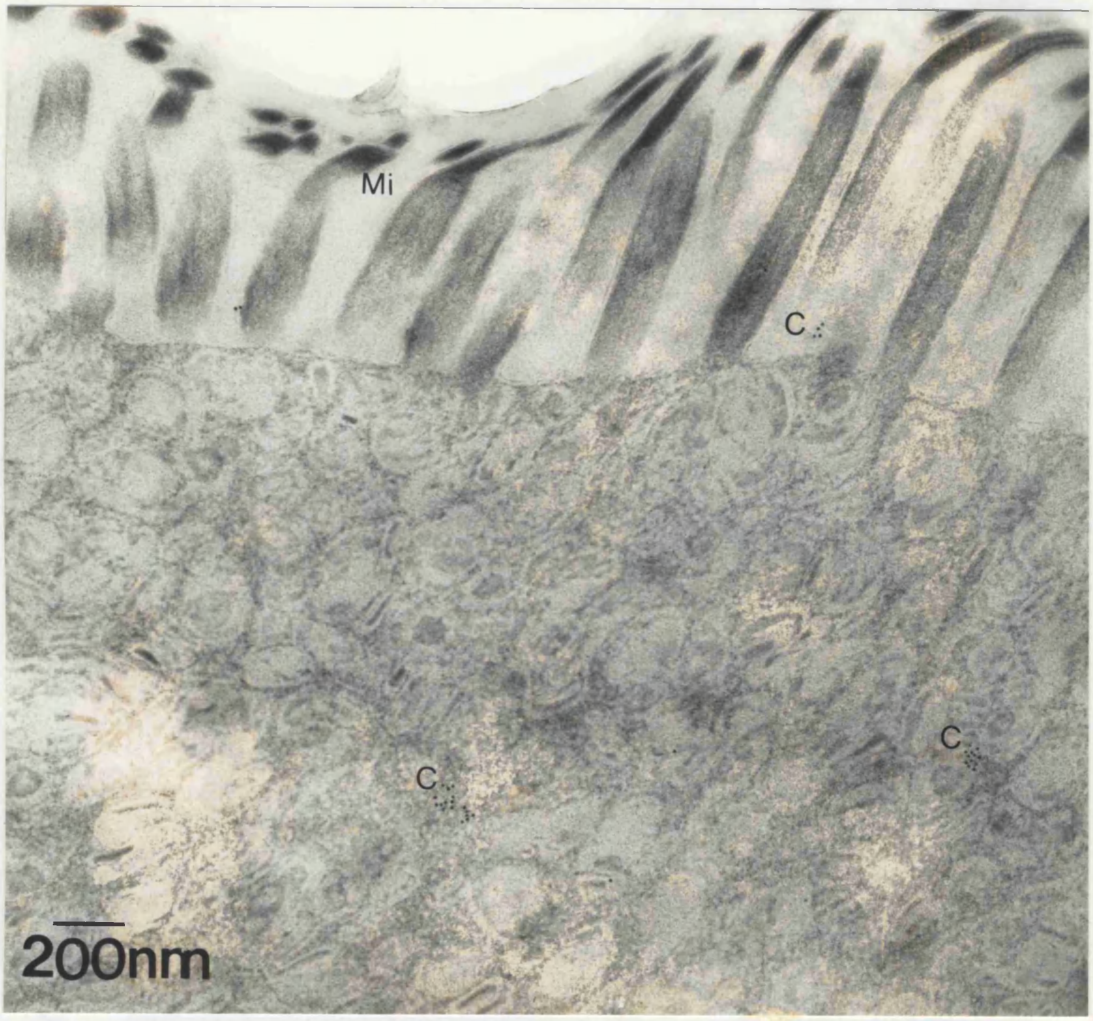


Figure 91. The Distal Cytoplasm

Calmodulin is associated with the discoid granules of the distal cytoplasm and with the base of the microthrix. This tissue was fixed with gluteraldehyde and is from the tail region. D=distal cytoplasm, C=calmodulin and Mi=microthrix. x25k.

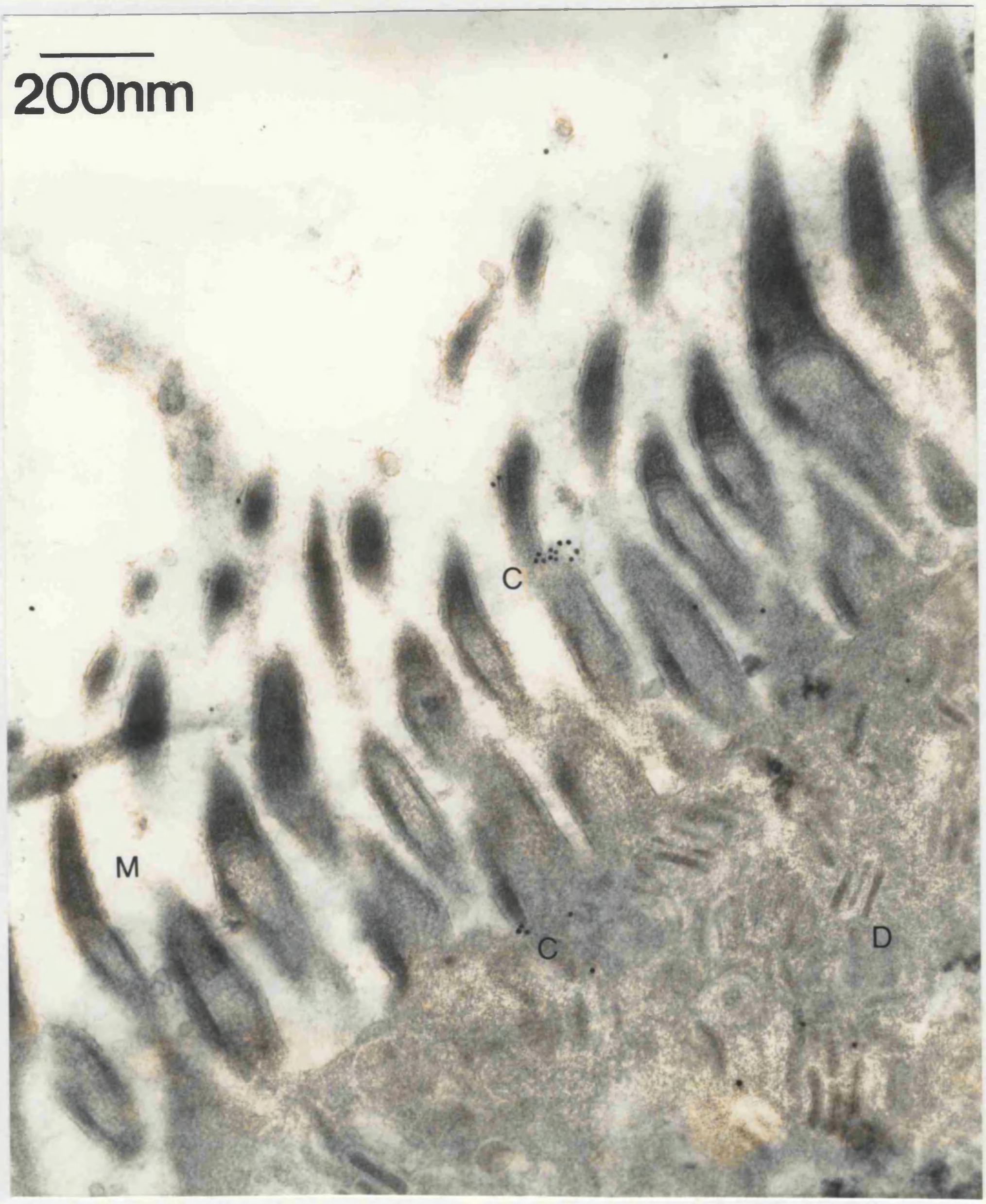


Figure 92. Microthrix From The Head Region

This shows calmodulin associated with the microthrix and distal cytoplasm of a section from the head region where the tissue was fixed with gluteraldehyde and osmium followed by treatment with tannic acid. Mi=microthrix, C=calmodulin and D=distal cytoplasm. x40k.

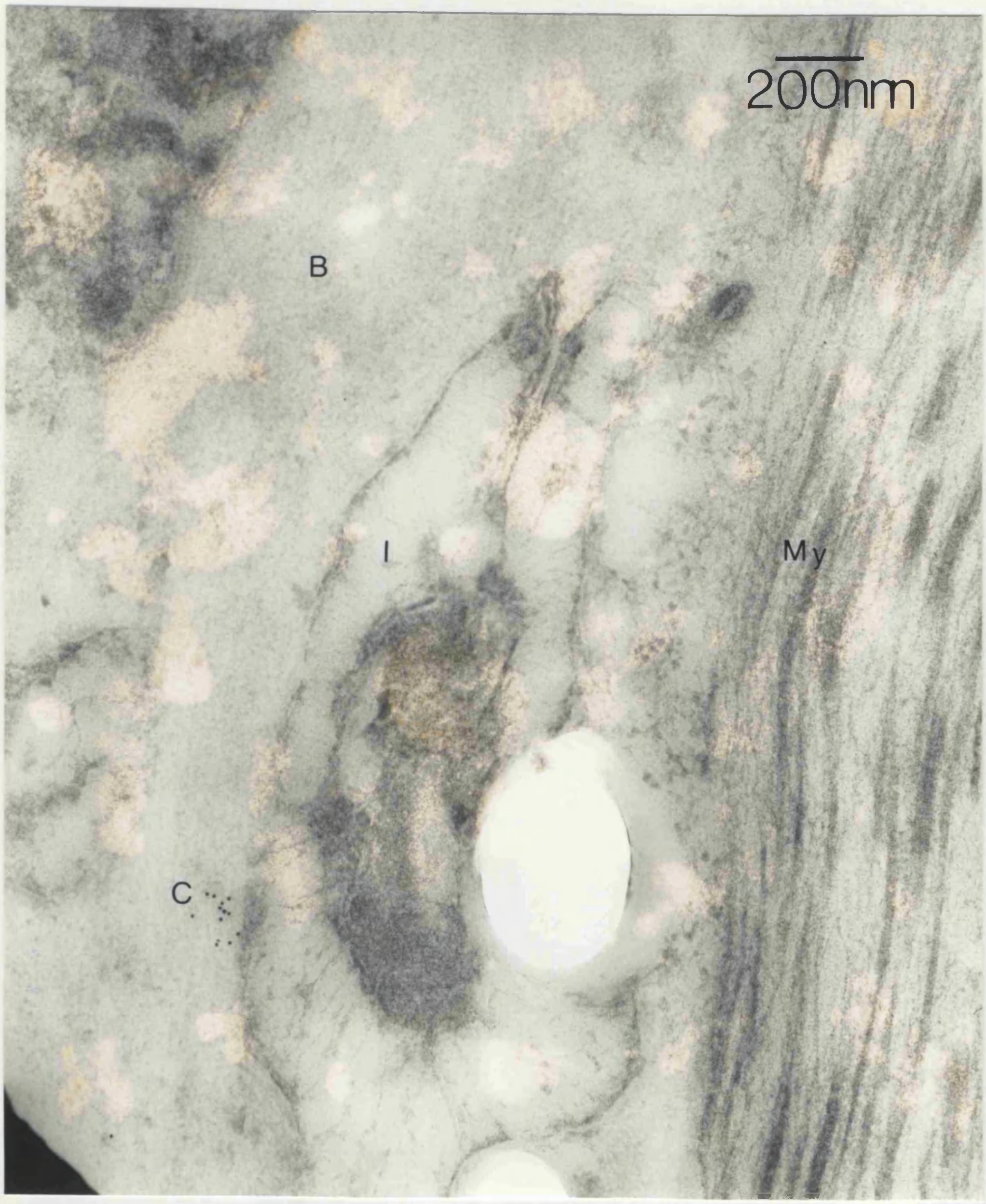


Figure 93. The Basal Lamella

This section from the tail region of tissue fixed with gluteraldehyde and shows a cross section of an internuncial process. Calmodulin label can be seen in the cytoplasm immediately adjacent to the process. I=internuncial process, C=calmodulin, B= basal lamella, D=distal cytoplasm and My=myofibres. x30k.

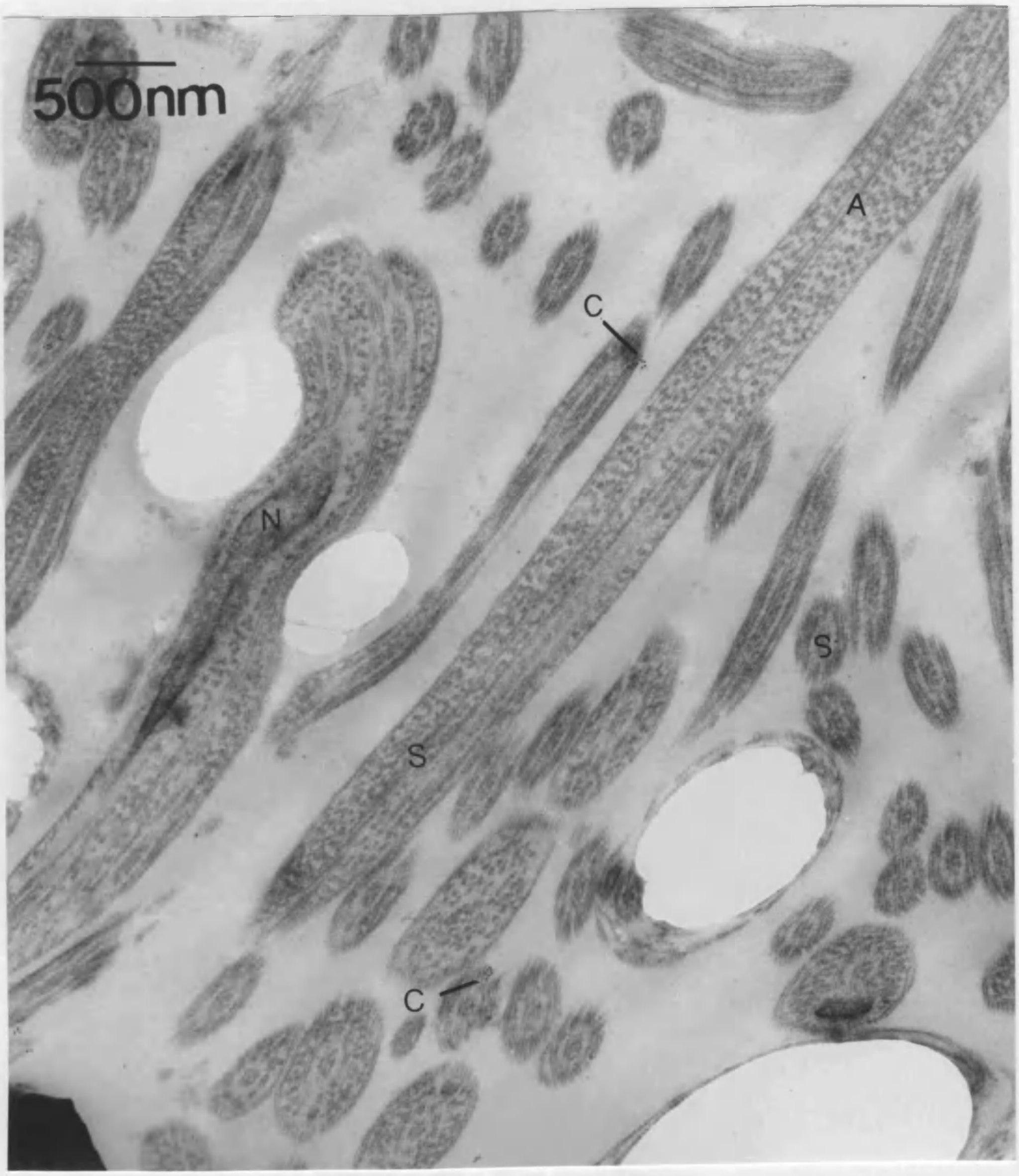


Figure 94. Spermatozoa

Calmodulin is associated with spermatozoa in this section from the middle region of the tapeworm. The tissue was fixed with gluteraldehyde. S=spermatozoa, C=calmodulin and A=axoneme. x15k.

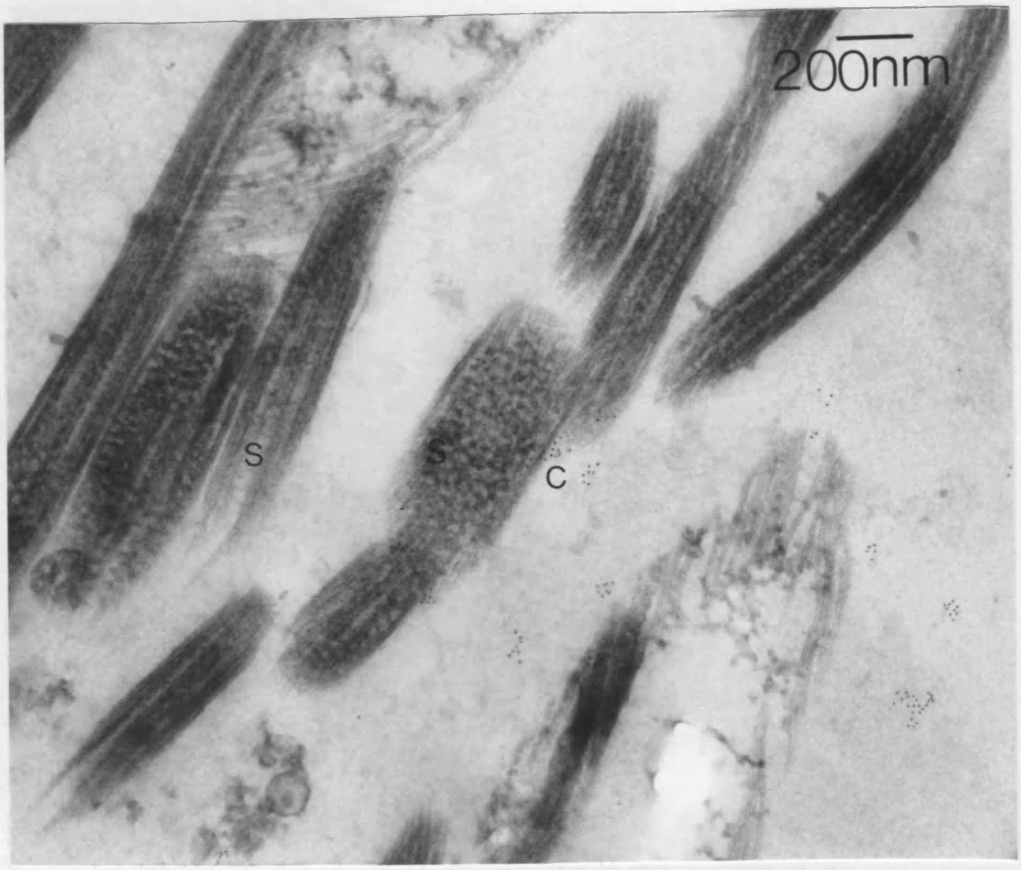


Figure 95. Spermatozoa

This shows a section from the middle region of the tapeworm with calmodulin associated with spermatozoa and the 'tissue' in which they are situated. Unlike other sections this one was treated with antibody diluted 1-5. The tissue was fixed with gluteraldehyde. S=spermatozoa, C=calmodulin. x20k.

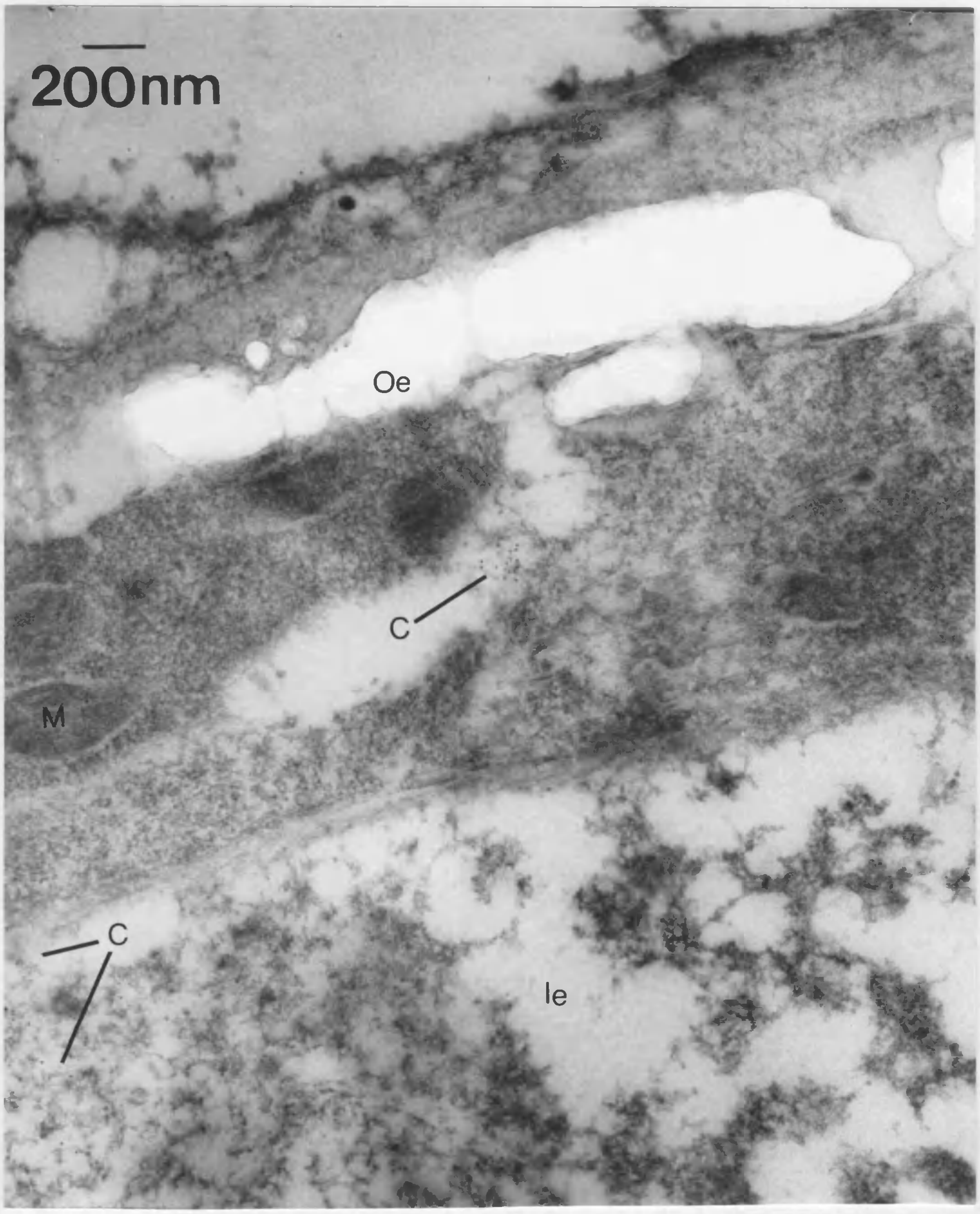


Figure 96. Envelopes of an Oncosphere ?

Calmodulin is localised to what appears to be part of the envelopes which form the oncosphere. However, identification is extremely difficult as the nature of these envelopes changes as the oncosphere matures. The section is from the middle region of the tapeworm and the tissue was fixed with gluteraldehyde. Oe=outer envelope, C=calmodulin, and Ie=inner envelope. x20k.

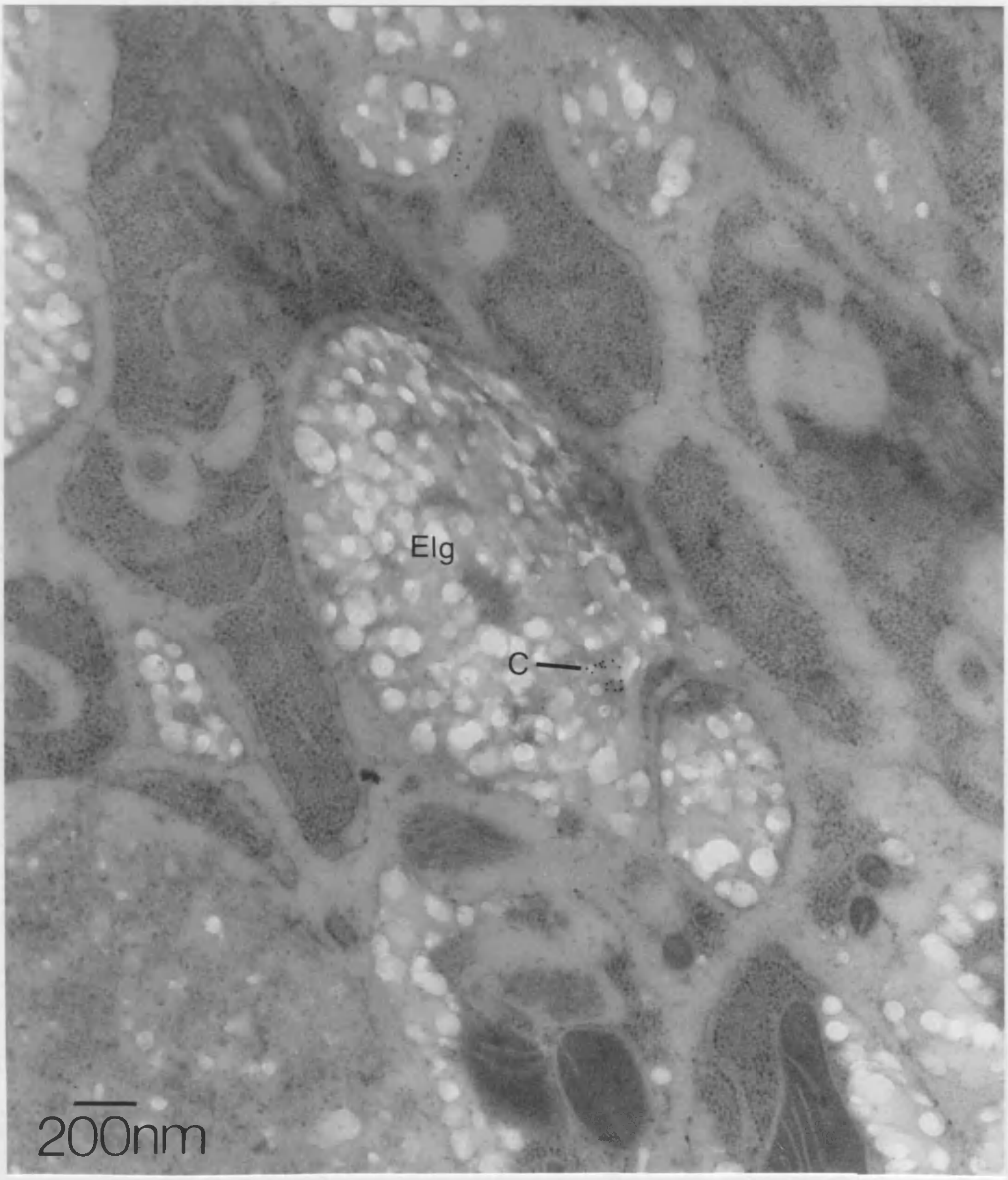


Figure 97. Electron Lucent Granules From the Tail Region

This section from the tail region shows calmodulin associated with an organ which appears to consist of electron lucent granules. These structures were only observed in the tail region and no corresponding structures could be found in the literature. This tissue was fixed with gluteraldehyde. Elg= electron lucent granules and C=calmodulin. x20k.

4 Discussion

The overall aim of this project was to discover more about calmodulin within the cestode Hymenolepis diminuta. In order to achieve this three different approaches were used: molecular biology protein biochemistry and immunocytochemistry. Each approach yielded different insights into the nature of the tapeworm and about calmodulin within the cestode.

4.1 Molecular Biology

The tapeworm proved to be a difficult organism from which to obtain clean DNA that was suitable for use to form a genomic library. The most probable cause of this difficulty is the presence of extensive glycogen stores within Hymenolepis diminuta which can represent up to 50% of the dry weight. Furthermore whilst 60-70% of the glycogen has a molecular weight of 25-60 million, 30% has a molecular weight of 900 million (section 1.2.2.2.). Glycogen tends to co-purify with the DNA wrapping around it and consequently causing shearing of the DNA. The glycogen could be digested, however, the experimental conditions required would also provide conditions in which endogenous nucleases could digest the DNA.

The presence of large stores of glycogen probably explains why the CTAB extraction method (section 2.2.1.2.) was the most successful. This method was originally devised to remove large quantities of polysaccharides found to contaminate DNA samples from plant tissue. Besides being very good at removing polysaccharides the CTAB method is routinely used to isolate cestode DNA because it also avoids repeated phenol extraction's which can result in losses of DNA (Hyde 1990).

When large sized DNA was obtained from H.diminuta, it was successfully digested by Sau3A. This would have produced suitably sized fragments of DNA that could have been ligated into BamHI sites within a lambda replacement vector, which in this case would have been EMBL3. This would then have been used to form a genomic library using lambda phage. However, after performing the various experiments to assess the optimum conditions, there was insufficient DNA on which to perform the final optimized experiment. Consequently, a genomic library was not produced.

Amplification of the calmodulin gene, using clean DNA, by the polymerase chain reaction was a successful alternative approach to forming a genomic library. The calmodulin gene was amplified and hybridized to the oligonucleotide probe for the calmodulin gene. This showed that there was probably only one gene copy of the calmodulin gene.

Many other organisms also only contain one gene copy for calmodulin, for example Drosophila melanogaster (Smith et al. 1987) and Ceanorhabditis elegans (Salvato et al. 1986). When the numbers of gene copies for calmodulin are compared it becomes apparent that as organisms become more complex, the number of gene copies increase (section 1.1.2. Table 3.). Most of the Protozoa posses one gene copy with the exception of Trypanosomes, which have only one gene arranged in tandem repeats (Tschudie et al. 1985). The Nematoda also have one gene copy, and evidence from this study suggests that the Platyhelminthes may also only possess one gene copy. Members of the Echinodermata and Chordata have two gene copies although the class Aves may only contain one functional gene and some members of the class Mammalia within the Chordata contain three functional genes. To date, calmodulin *per se* has not been found in the prokaryotes but calcium binding proteins with very similar structures to calmodulin do exist. However, these are generally composed of 177 amino acids instead of the 148 amino acids found in calmodulin (Swan et al. 1987).

There were many problems involved in trying to insert the PCR product into either of the two vectors that were selected (M13 and pUC). It seems likely that this was largely due to an unfortunate run of luck. PCR DNA is difficult to insert into vectors because it is not phosphorylated. Consequently, the vectors have to be phosphorylated and occasionally the PCR DNA will not ligate successfully. To overcome this the PCR DNA can be phosphorylated and the vector dephosphorylated. However, the procedures involved result in some loss of the initial starting material. Consequently, as there was a limited supply of starting material this was not a favourable option. Another factor that hindered the ligation procedure was that one of the ligase enzymes used during the work, appeared to be working sub-optimally. But, despite all of these problems some positive transformants were obtained.

Another trait of PCR DNA is that once inserted into the vector it becomes resistant to digestion by restriction enzymes the consequence of which is that the insert cannot be extracted from the vector. It can be seen that using PCR DNA has many pitfalls, however, when successful, it is a very rewarding methodology to use.

It was due to the problems encountered with the PCR approach that the alternative route of obtaining mRNA and using it as a template to produce cDNA library was tried. Isolation of mRNA gave the option of not only producing cDNA but also of investigating the translation products within an expression system such as Xenopus laeviis. It was found that it was far easier to obtain good preparations of RNA from the cestode material than it had been to obtain the DNA. The isolation of mRNA was also successful, although the quantities obtained were somewhat low. However, there was sufficient to perform cDNA synthesis, but insufficient to perform any translation studies.

Overall, the molecular biology proved an unsuccessful approach in relation to the available resources and the length of time of the project. Consequently, this approach was shelved in the hope that it would be possible to recommence it at a later date.

4.2 Protein Biochemistry.

Instead of trying to isolate the gene for calmodulin, it was decided to isolate the protein, which could then be sequenced and used in further investigative studies. Four main isolation methods were tried in developing an isolation procedure for H.diminuta, all of which utilized the hydrophobic phenyl Sepharose column, as used by Gopolakrishna and Anderson (1982), as one of the principle isolation steps. It became evident that the success of the method used to isolate calmodulin depended on the nature of the tissue being processed.

Gopolakrishna and Anderson (1982) originally used the phenyl Sepharose method to isolate calmodulin from bovine brain. These workers applied tissue homogenate that had either undergone an iso-electric precipitation with ammonium sulphate or that had just been homogenized and centrifuged to a phenyl Sepharose column. On application of the EGTA buffer a single peak was produced which they found contained calmodulin. However, these workers failed to give any data concerning the yield of calmodulin.

In this study, tissue was treated with a heat treatment step or an ammonium sulphate precipitation step, or a combination of TCA precipitation and ammonium sulphate precipitation steps before application to the phenyl Sepharose column. In the majority of cases only a single peak occurred. However, on some occasions the calmodulin bound very tightly to the column, so that it could only be removed by the application of 6M urea. It has been reported that the temperature of the column can affect the efficiency with which the column initially binds proteins (Gopolakrishna & Anderson 1982). During the course of determining a suitable isolation method, in this study, it became apparent that not only did the temperature interfere with the affect of binding, but also the speed at which the protein mixture was applied to the column. When the phenyl Sepharose was first tried using pig thymus it was run under gravity, which is equivalent to 2ml per minute. This resulted in three peaks forming on the application of the EGTA buffer. The first peak contained a wide range of proteins and the second and third peaks contained calmodulin. However, when the column was slowed to 0.5ml per minute only one peak emerged which contained calmodulin and some contaminating proteins.

An isolation procedure using the following steps: homogenization, heat treatment, ion-exchange chromatography and gel filtration is reported to remove 98% of the initial protein (Dedman & Kaetzel 1983). In this report a modified version of Gopolakrishna and Andersons method was also used where a heat treatment step was incorporated before phenyl Sepharose chromatography. This was then followed by DEAE chromatography to remove contaminating proteins from the calmodulin peak obtained from the phenyl Sepharose. Using this method a homogeneous preparation of calmodulin was obtained (Dedman & Kaetzel 1983). This modified method has also been used to isolate calmodulin from plants. However, workers have found that using an ammonium sulphate precipitation step instead of the heat treatment step, gives better results. This is probably because plant tissue has a low protein content relative to fresh weight and it contains a high level of protease's, quinones, phenolics and pigments that are more readily extracted by precipitating the tissue with ammonium sulphate (Anderson 1983).

Some workers prefer not to use the heat treatment step for fears of increased proteolytic degradation whilst the temperature is rising. Also, some workers have reported significant losses because calmodulin becomes trapped in the large precipitates which form on heating (Charbonneau et al. 1983). For eaxmple, when trypanosome calmodulin was isolated the heat treatment step was omitted and DEAE-52 chromatography performed before phenyl Sepharose chromatography. A similar method has also been used to isolate bovine brain calmodulin, but when isolating calmodulin from red erythrocytes an ammonium sulphate precipitation step was included before the DEAE chromatography (Ruben, Egwuagu & Patton 1983).

Another modified version of Gopolakrishna and Anderson (1982) has been used to isolate bovine brain calmodulin. This included ammonium sulphate precipitation and DEAE-cellulose chromatography before phenyl sepharose chromatography (Charbonneau et al. 1983). However, it was found that the calmodulin fraction was contaminated by a protein that was thought to be S-100, which is also a calcium binding protein and has been found to contaminate many isolated preparations of calmodulin when calcium dependent affinity columns have been used. These include phenyl Sepharose, fluphenazine-Sepharose and CAPP-Sepharose (CAPP=2-chloro-10-aminopropyl-phenothiazine). It is also not the only contaminating protein workers have found when isolating calmodulin, for eaxmple when isolating calmodulin from Chlamydomonas with CAPP-Sepharose a protein with a molecular weight of 26kD was found. Similarly, when peanut calmodulin is isolated it is frequently contaminated with low molecular weight polypeptides of 7 to 8 kD, all of which partially inactivate the calcium dependent ATPase. Many of these polypeptides are thought to arise because of proteolysis (Charbonneau et al. 1983).

A combination of ammonium sulphate precipitation and DEAE chromatography followed by Sephadex G-100 gel filtration has also been used instead of phenyl sepharose chromatography to isolate calmodulin from bovine brain (Teo, Wang & Wang 1973). It was found that approximately 80% of the total protein in the original sample was removed before gel filtration with a total of 1.2mg of calmodulin being recovered from an original sample containing 65g protein. This was calculated to be a 14% recovery based on the activity of calmodulin (Teo, Wang & Wang 1973). However, it is only 0.002% of the total protein, which is considerably lower than the yields obtained during this study, which ranged from 0.06% to 13%.

Using a trichloroacetic acid precipitation step in the isolation procedure has also been widely used. For example it has been used to isolate Tetrahymena and sea anemone (Metridium senile) muscle calmodulin (Takagi et al. 1980; Yazawa et al. 1981). Some workers have used a combination of trichloroacetic acid precipitation followed by DEAE chromatography and fluphenazine-Sepharose chromatography where the overall yield of calmodulin was apparently 70%, but, there is no reference whether this is based on activity or protein content (Charbonneau et al. 1983).

Originally calmodulin was isolated whilst isolating phosphodiesterase. The method involved homogenizing brain tissue, ammonium sulphate precipitation, calcium phosphate gel and DEAE-cellulose chromatography (Cheung 1970). The calmodulin was found to elute after the phosphodiesterase on the DEAE-ion exchange column. However, there are no details concerning the recovery of protein. By the 1980's this method had been modified so that tissue homogenates were treated with a heat treatment step followed by chromatography on a fluphenazine-sepharose column (Wallace, Tallant and Cheung 1980). However even this method was further modified to produce a two-step isolation procedure with the first step on either a fluphenazine or phenyl sepharose column and the second step with gel filtration on Sephadex G100 (Wallace, Tallant & McManus 1987).

To obtain bovine testes' calmodulin a method based on hydrophobic chromatography was used in which tissue was subjected to phenyl sepharose chromatography and

DEAE-Sephacel chromatography before high resolution gel permeation chromatography on a Bio-Sil TSK-125 column (Wasco, Kincaid and Orr 1989).

Two lesser used methods have been used to isolate calmodulin from paramecium. The first method isolates calmodulin in one step, by monoclonal antibody affinity chromatography and produces calmodulin that is greater than 99% homogeneous. The second method uses phenyl sepharose chromatography followed by HPLC anion-exchange column chromatography (Mono-Q), with a final purification step using a melettin affinity column (Evans and Nelson 1989).

A calmodulin-like protein has been isolated, using DEAE-ion exchange chromatography followed by phenyl sepharose chromatography, from the cyanobacteria (also referred to as blue/green algae), Anabaena *sp.* (Bianchini et al. 1990). When this was analysed by SDS-PAGE, the protein showed as a band with a molecular weight of 58kD, with two minor bands of 40 and 16kD. The calmodulin like protein activated the endogenous adenylate cyclase that had previously been shown to be stimulated by bovine calmodulin. Furthermore the Anabaena *sp.* calmodulin-like protein could also activate bovine phosphodiesterase. Calmodulin like proteins have been reported in other bacteria however, they have been found to contain more amino acid residues than calmodulin. For example in Streptomyces erythraeus the calmodulin-like protein was composed of 177 amino acids, with a molecular weight of 20kD, whereas calmodulin is composed of 148 amino acids. It has also been reported that the amino acid composition is completely different from calmodulin although the calcium binding sites share some similarity (Swan et al. 1987).

As has already been stated, during these studies the protein recovered in the calmodulin fraction has ranged from 0.06% to 13% depending of the isolation method used and the tissue under investigation. In method one, tissue was heat treated, subjected to phenyl-Sepharose chromatography followed by DEAE chromatography. When this method was used to isolate pig thymus calmodulin the calmodulin fraction contained between 0.3 and 0.8% of the total protein. However when it was used to isolate cestode calmodulin it contained 3% of the total protein.

In method 2, tissue was subjected to ammonium sulphate precipitation before phenyl sepharose and DEAE chromatography. When pig thymus calmodulin was isolated using this method, the calmodulin fraction contained 13% of the original homogenate protein. However, when it was used to isolate cestode calmodulin the method worked unsatisfactorily. Method 3 involved trichloroacetic acid precipitation followed by ammonium sulphate precipitation before phenyl sepharose chromatography. Pig thymus resulted in a calmodulin fraction containing 2.7% of the original protein but, as with method 2, when used to isolate cestode calmodulin the method did not work satisfactorily. Using method 4 to isolate pig thymus calmodulin resulted in the lowest percentage recovery, being 0.06%, but the calmodulin fraction was clear of contaminating proteins. This method resulted in a cestode calmodulin fraction that contained 0.41% of the total protein, but it was contaminated.

Calmodulin samples containing 0.02% of the total protein have been reported from rat testes, so the yields obtained from all the methods tried in this study were very good (Means et al. 1991). However, the results show that the best isolation methods for pig thymus are either method 2 or method 4 whilst for tapeworm only method 1 appeared to give a pure homogenous preparation of calmodulin. This difference in the most suitable method is presumably due to the difference in the initial protein mixture contained in the homogenate. In tapeworm 98% of the protein appeared to be soluble whilst in pig thymus only 50% of the protein was soluble. From this, one can deduce that the most suitable isolation method for calmodulin from the rat testes, which also contained ≈98% soluble protein, would also be method 1. Furthermore these findings support other workers' opinions that the best isolation method for calmodulin depends very much on the tissue being processed.

Having obtained samples of calmodulin it was tested for its biological activity using the phosphodiesterase assay, as detailed in section 2.3.4. Initial experiments found that calmodulin stimulated the enzyme's activity by over 50%. However, after several experiments it was obvious that the phosphodiesterases own basal activity was increasing, with decreased sensitivity to calmodulin. It was obvious that this inconsistency was not due to the samples of calmodulin because even the Sigma calmodulin failed to activate the phosphodiesterase. A supplier of phosphodiesterase, Pharmacia, reported that phosphodiesterase is unstable once made into a solution from its lyophilized state. Pharmacia claimed that the enzyme was only stable to calmodulin for approximately six hours once prepared in solution, and that thereafter the enzymes basal activity increased with a concomitant decreased sensitivity to calmodulin.

This hypothesis was supported by the calmodulin sensitivity experiment with phosphodiesterase. It was found that there was a gradual decrease in the interaction between phosphodiesterase and calmodulin-Sepharose over time and that within 24 hours the interaction between phosphodiesterase and calmodulin was non-specific.

It has been reported that phosphodiesterase can be isolated and stored in a frozen state, at -70°C, for upto one year without significant loss of its activity providing the enzyme is not subjected to repeated freezing and thawing (Wallace, Tallant & Cheung 1983). However, the isolation procedure used to obtain the phosphodiesterase is apparently crucial, as it can affect the enzymes basal activity and its stability (Kincaid & Vaughnan 1986). The storage of the enzyme at low protein concentrations can result in a gradual decline in sensitivity to calmodulin, with no loss in its basal activity or total activity with no sign of enzyme degradation. Furthermore, Ferguson plot analysis of adsorption electrophoresis has shown that changes in the observed phosphodiesterase activity are due to a shift from a dimeric form (120-140kD) to a monomeric form (50-60kD). Only the dimeric form is sensitive to calmodulin stimulation (Kincaid & Vaughnan 1986).

Apparently, the purity of the phosphodiesterase also affects the effects of calmodulin. When phosphodiesterase is highly purified, calcium/calmodulin serve to stabilize the enzyme, whilst in a less purified preparation they serve to destabilize it. Furthermore, ammonium ions, imidazole, vitamin E derivatives and lysolecithin will also activate phosphodiesterase, whilst the presence of potassium ions will inhibit the basal activity without affecting calmodulin sensitivity (Lin & Cheung 1980).

Overall it has been concluded that certain purification and storage conditions, e.g. low pH, high ionic strength, affects the ability of phosphodiesterase to self-associate at low protein concentrations and thereby alter its basal activity. However, the ability to interact with calmodulin is retained, but may not be activating except when binding to a dimeric form of phosphodiesterase (Kincaid & Vaughnan 1986).

The phosphodiesterase assay has been widely used, despite problems with enzyme stability, because it will only detect calmodulin that is biologically active. It will not detect calmodulin that has been rendered biologically inactive or is present in a complex with another calmodulin binding protein. Sometimes the phosphodiesterase assay has been used to quantitate the levels of calmodulin in a sample. However, it has been shown that the assay can underestimate the quantity of calmodulin (Wallace, Tallant & Cheung 1983).

Generally, 5'-nucleotidase from snake venom is used to perform the removal of adenosine and phosphate from the 5'-AMP rather than alkaline phosphatase. However, if the phosphodiesterase and the 5'-nucleotidase are included together at the start of the reaction, then the phosphodiesterase will be irreversibly stimulated by proteolytic enzymes present in the snake venom (Wallace, Tallant & Cheung 1983). This will then render the phosphodiesterase insensitive to calmodulin. In this study, the two reactions were always performed sequentially, so it seems unlikely that any proteolytic enzymes present in the alkaline phosphatase were responsible for the observed results.

There are also a number of agents that can interfere with the phosphodiesterase experiment. These include the presence of phosphodiesterase, calmodulin binding proteins, proteolytic enzymes and high levels of lipid in the calmodulin sample (Wallace, Tallant & Cheung 1983). The presence of any proteolytic enzymes, e.g. calpain, will result in limited proteolysis resulting in the irreversible stimulation of phosphodiesterase, with a concomitant loss of stimulation by calmodulin. It is thought that limited proteolysis of phosphodiesterase and calmodulin-binding enzymes either removes the calmodulin binding domain or both the calmodulin-binding and inhibitory domains. In either case the inhibition is relieved and the enzyme expresses its full activity, which is no longer calmodulin sensitive (Lin and Cheung 1980; Wang, Villobo & Roufogalis 1989). Inclusion of proteolytic inhibitors is possible, but they frequently bind avidly to calmodulin and so render it useless, e.g. aprotinin. The presence of any lipids also stimulates phosphodiesterase, decreasing its sensitivity to calmodulin but this can be overcome by the addition of 0.02% (v/v) of a non-ionic detergent. It is also possible for there to be an apparent loss of calmodulin through non-specific binding to the tubes used for the assay (Wallace, Tallant & Cheung 1983; Wang, Villobo & Roufogalis 1989). However, this can be minimised by treating the tubes with 0.1% (w/v) bovine serum albumin.

Whilst it is feasible that there may have been either calmodulin binding proteins or endogenous phosphodiesterase present in the calmodulin sample, the same samples were used in the initial experiments, where calmodulin did stimulate the phosphodiesterase, as were used in later experiments where calmodulin failed to stimulate phosphodiesterase. Also, this does not explain why the Sigma calmodulin failed to stimulate the phosphodiesterase after initially stimulating it, as the Sigma calmodulin appeared as a single homogenous band on SDS-PAGE.

The degree of calmodulin stimulation of phosphodiesterase appears to vary widely. It has been suggested that these differences are due to the presence of other phosphodiesterases and activator substances, the conditions used in the assay and the contribution of proteolytically activated forms of the enzyme (Kincaid & Vaughnan 1986). However, it has been found that if a stabilizing protein such as bovine serum albumin or ovalbumin is included in the reaction more reproducible results are obtained (Kincaid & Vaughnan 1986).

The phosphodiesterase used in the experiments in this study was obtained from a commercial supplier consequently one would expect a homogeneous preparation free of contaminating proteins. But, the data sheet supplied with the enzyme showed that the phosphodiesterase contained small percentages of various proteins these included 5'nucleotidase, 5'ATPase, inorganic pyrophosphatase, alkaline phosphatase and phosphodiesterase 2',3'-cyclic nucleotide. The levels of these impurities ranged between 0.02 and 0.25% of the activated phosphodiesterase activity. However there are no protease's, so it seems unlikely that the observed results, in these studies, are due to the effects of proteolytic enzymes' in the phosphodiesterase. It is possible that there were proteolytic enzymes present in the calmodulin samples isolated from either tapeworm or pig thymus that could have activated the phosphodiesterase but, the commercially supplied calmodulin also failed to stimulate phosphodiesterase. Therefore, the most probable explanation is that the phosphodiesterase had disassociated into the monomeric form so that it was insensitive to calmodulin activation.

Although initial experiments had proved that the isolated calmodulin stimulated phosphodiesterase, there was still a question mark over whether the samples actually contained calmodulin and if it was biologically active. To further verify the identity of the isolated protein both ELISA's and western blots were performed. The ELISA would detect calmodulin, and prove that the protein had retained its antigenicity, whilst the western blot would reveal the identity of the protein to which the antibody used in the ELISA was binding. The ELISA worked perfectly and western blot analysis revealed antibody binding to calmodulin. However, in some instances, there appeared to be three bands instead of the one visible after staining the duplicate half of the gel with Coomassie blue. The most probable reason for this is that the antibody was binding to different forms of calmodulin within the sample, i.e. calmodulin without calcium, and calmodulin with either 1, 2, 3 or 4 calcium ions bound, all of which will migrate to slightly different degrees.

There have been few reports on the use of ELISA's and western blots for detecting calmodulin. Antibodies to Tetrahymena calmodulin have been used to identify other calmodulin samples in ouchterlony immunodiffusion tests. The tetrahymena antibody formed a strong precipitin line against Tetrahymena calmodulin and Paramecium calmodulin but it did not react with porcine, sea-urchin, sea anemone and slime mould calmodulin (Suzuki et al. 1982). Antibodies to trypanosome calmodulin have also been produced and used to identify other sources of calmodulin. The trypanosome calmodulin was found to be very specific for trypanosome calmodulin as it failed to react to bovine calmodulin (Ruben et al. 1984). It can be seen, that antibodies raised to calmodulin can be very species specific.

The anti-calmodulin used during this project was either raised in goat or sheep. The goat antibody was raised to bovine calmodulin and was reported to recognise calmodulin from bovine brain and calf thymus whilst the anti-calmodulin, raised in sheep, supplied by Calbiochem, recognised calmodulin from bovine testes and heart, human erythrocytes, rabbit heart and liver, and rat heart and liver. Consequently both antibodies would be expected to recognise the Sigma bovine calmodulin, used as a control, but there was a possibility that they may not recognise calmodulin from either pig thymus or tapeworms. Fortunately, the results show that the calmodulin antibodies did recognise both cestode and pig thymus calmodulin. From this it can be inferred that cestode, pig thymus and bovine calmodulin all share, at least, one common epitope.

Having established the identity of the isolated protein as calmodulin it was hoped that some of the cestode calmodulin could be used to prepare a calmodulin-Sepharose or agarose column, which could then be used to isolate the calmodulin binding proteins. However the quantities of calmodulin required to perform this coupling, were not readily available. Fortunately, at this time, calmodulin sepharose and agarose became commercially available, so this was used to isolate calmodulin binding proteins.

The results of these studies showed that the cestode contained several calmodulin binding proteins. It was found that there were two calmodulin binding proteins with molecular weights of 205 and 45kD that bound calmodulin in a calcium dependent and independent manner. As they were also eluted when the homogenate was treated with and without detergent, it seems probable that these two proteins are present in the cytosol and membrane bound. Beside these two proteins there were around ten other calcium-dependent calmodulin binding proteins. These had molecular weights of 116, 97, between 66 and 97, 66, between 45 and 66, between 36 and 45 and around 20kD. When detergent was included only one additional calcium dependent calmodulin binding protein was isolated, which had a molecular weight of between 116 and 205kD. As this was isolated only in the presence of detergent it is obviously a membrane bound protein.

Calcineurin, a calmodulin binding protein (CaM-BP), is reported to constitute around 30% of the CaM-BP's isolated by calmodulin affinity chromatography (Kincaid & Vaughan 1988). It has a molecular weight of 80kD by gel filtration, but on SDS-PAGE it appears as two subunits, an α-subunit of 60kD and a β-subunit of 19kD. Therefore, the protein isolated with a molecular weight of 60kD could be the α-subunit of calcineurin, which contains the catalytic domain, several regulatory domains including the β–subunit binding site, the calmodulin binding domain and a phosphorylation site. Calcineurin can be phosphorylated by either the calcium/calmodulin dependent protein kinase and protein kinase C (Calalb, Kincaid & Soderling 1990). It has also been reported that when calcineurin is phosphorylated it binds to calmodulin-Sepharose in a calcium dependent manner (Hashimoto, King & Soderling 1988). In this study this 60kD protein was also found to be calcium dependent. However, the calmodulin dependent phosphodiesterase also has a molecular weight of around 57-60kD, so this could also be the 60kD protein. Alternatively, it could be a mixture of both calcineurin and phosphodiesterase.

Using an ^{125}I-calmodulin gel overlay technique seven major calcium-dependent CaM-BP's with molecular weights of 64, 53, 36, 32, 28, 26 and 25kD were identified in Saccharomyces cerevisiae (Liu et al. 1990). By comparison the soluble CaM-BP's were also isolated with a calmodulin-Sepharose column. When these were analysed by SDS-PAGE five additional CaM-BP's were isolated, three proteins with molecular weights greater than 72kD, and two proteins with molecular weights of 68 and 60kD. The CaM-BP's of 64, 53, 35, 32, 28 and 26kD were found to be soluble whilst those of 54, 40, 34, and 28kD were membrane bound (Liu et al. 1990). Furthermore the nuclear CaM-BP's have also been identified and characterized using an ^{125}I-calmodulin overlay technique of total nuclear proteins analysed by SDS-PAGE (Hiraga et al. 1993). Some proteins were found to bind to calmodulin in a calcium independent manner with molecular weights between 14 and 18kD. It was proposed that these were histones, which are one of many known CaM-BP's known to bind calmodulin in a calcium independent manner. Some other proteins with molecular weights of between 24-31kD were also identified, which bound to calmodulin in a calcium independent manner. There was one protein with a molecular weight of 43kD that bound calmodulin in both the presence and absence of calcium. However, there were twelve calcium-dependent CaM-BP's with molecular weights of: 200, 100, 66, 62, 44, 42, 38, 36, 34, 32, 27 and 25kD (Hiraga et al. 1993). The CaM-BP's of the nuclear fractions of yeast were also isolated on a calmodulin Sepharose column, and then analysed using the ^{125}I-calmodulin gel overlay techniqu. On this occasion the calcium independent CaM-BP's found in the primary study were absent (Hiraga et al. 1993).

A total of 25 CaM-BP's were identified in Dictyostelium discoideum during cell fusion using an ^{35}S-VU-1-calmodulin overlay of the total proteins, which had been analysed by SDS-PAGE (Lydan & O'Day 1993). In cells where sexual development was occurring calcium dependent CaM-BP's with molecular weights of 155, 91, 85, 48, 46, 38 and 28 kD were found. There were also three calcium-independent CaM-BP's with molecular weights of ≈50, 40 and 35kD. The binding of calmodulin to these proteins was inhibited when trifluoroperazine was included with the ^{35}S-VU-1-calmodulin. It was thought that the protein with a molecular weight of 155kD might be α-spectrin that has a molecular weight of 160kD, whilst β-spectin has a molecular weight of 158kD. Spectrin has been detected in many cell types and has the capacity to interact with F-actin, lipid membranes and calmodulin. Spectrin is also obligately required for fusion of chromaffin granules with the plasma membrane, during the secretion of catecholamines (Lydan & O'Day 1993).

An unidentified 81kD CaM-BP has been isolated from bovine brain using three chromatographic steps: anion exchange, gel filtration and calmodulin affinity chromatography (Tokumitsu et al. 1991). The protein is phosphorylated by protein kinase C and cAMP dependent protein kinase and has a highly acidic nature with a pI of 4.3.

The CaM-BP's present in hair bundles have been investigated following the discovery that calmodulin is concentrated near their stereociliary tips (Walker, Hudspeth & Gillespie 1993). The hair bundle is the receptive organelle for mechanoelectrical transduction in vertebrate hair cells and is composed of dozens of stereocilia (cylindrical processes derived from microvilli), which project from the cellular apex in staggered ranks. It has been proposed that calmodulin may be involved in the process of adjusting the position of sensitivity of hair bundles, a process referred to as 'adaptation', which is controlled by the calcium concentration within the stereocilia. Six major calcium-dependent CaM-BP's were identified with molecular weights of 25, 35, 145, 175, 240 and 350kD and 5 minor ones with molecular weights of 33, 36, 57, 107 and one that was greater than 350kD (Walker, Hudspeth & Gillespie 1993).

When the nuclear replitase multienzyme complex of CHEF/18 cells was analysed by SDS-PAGE and probed with calmodulin conjugated to alkaline phosphatase, two calcium-dependent CaM-BPs with molecular weights of 68 and 120kD were found (Subramanyam et al. 1990). The replitase complex is responsible for nuclear DNA replication during S-phase consequently it was proposed that calmodulin and the 68kD CaM-BP may be involved in maintaining the structural and/or functional integrity of the replitase complex. The 120kD CaM-BP is apparently similar to a CaM-BP found associated with DNA polymerase-α of mammalian cells (Hammond et al. 1988). Furthermore when immunopurified polymerase-α from calf thymus was probed, using an ^{125}I-calmodulin overlay technique, two CaM-BP's were found with molecular weights of 120 and 48kD (Hammond et al. 1988).

The CaM-BP's in liver nuclei have also been investigated using an ^{125}I-calmodulin gel overlay technique (Serratosa et al. 1988). In normal liver nuclei five CaM-BP's were identified that had molecular weights of 60, 65, 120, 130 and 150kD. By comparison, regenerating liver nuclei displayed increased quantities of the 130 and 150kD proteins, and decreased quantities of the 60, 65 and 120kD proteins. It was thought that the 130kD CaM-BP was myosin light chain kinase (Serratosa et al. 1988).

Further studies on the nuclei of rat liver also reported the presence of myosin light chain kinase together with caldesmon, actin and calmodulin (Bachs et al. 1990). In quiescent liver nuclei two major proteins with molecular weights of 130 and 240kD, together with three minor proteins with molecular weights between 50 and 60kD were identified. These proteins were identified by immunoblotting, as α-spectrin (240kD) and myosin light chain kinase (130kD). When the nuclei were solubilized with triton and the CaM-BP's isolated several 'new' calcium-dependent CaM-BP's were identified. The major protein isolated had a molecular weight of 62kD but there were several other proteins with molecular weights of >240, 240, 200, 180, 165, 145, 105, 94 and 72kD. The 72kD protein was identified as caldesmon, which has a molecular weight ranging between 71 and 77kD. Sometimes CaM-BP's with molecular weights of 120kD and 150kD were isolated but these were thought to be proteolytic products of α-spectrin, as when protease inhibitors (1mM PMSF) were included these two bands were not observed. The detection of α-spectrin within the nucleus is apparently somewhat surprising as it has been widely assumed that it was located exclusively to the plasma membrane. However, nuclear α-spectrin was observed associated with the nuclear membrane and some intranuclei granuli (Bachs et al. 1990).

Caldesmon has been isolated to near homogeneity using calmodulin-Sepharose (Hayashi et al. 1991). There are two iso-forms of caldesmon, *h*-caldesmon, which has a molecular weight of between 120 and 150kD, and *l*-caldesmon, which has a molecular weight of 70 to 80kD. *h*-Caldesmon is found predominantly in smooth muscle tissue whilst *l*-caldesmon is widely distributed throughout non-muscle tissues and cells. Both forms of caldesmon inhibit smooth muscle and non-muscle actomyosin ATPase but this inhibition is relieved by calcium/calmodulin (Hayashi et al. 1991).

The CaM-BP's of rod outer segments in frogs have been identified by performing an ^{125}I-calmodulin gel overlay of an SDS-PAGE of rod outer segment proteins (Nagao, Yamazaki & Bitensky 1987). Six calcium-dependent CaM-BP's were reported with molecular weights of 240, 140, (105), 67, 53, 47 and 35kD. Two of these, with molecular weights of 67 and 35kD, were also isolated in the presence of EGTA. The 35kD protein was identified as a monomer of rhodopsin, with the 67kD protein being the dimer. However, native rhodopsin (35kD) does not bind to calmodulin (Nagao, Yamazaki & Bitensky 1987).

The ^{125}I-calmodulin overlay technique has also been used to identify the CaM-BP's of human platelet proteins that have been western blotted onto nitrocellulose (Wallace, Tallant & McManus 1987). Ten calcium-dependent CaM-BP's were identified with molecular weights of 245, 225, 175, 150, 90, 82, 60 & 41kD but both the 82 and 41kD proteins appeared as doublets. The 245, 225 and 175kD CaM-BP's were only found in the particulate fraction of platelets, whereas all the other CaM-BP's were found in both the soluble and particulate fractions. A polyclonal antibody for myosin light chain kinase bound to the 225 and 90kD proteins. However, myosin light chain kinase has a subunit molecular weight of 150kD, so it is probable that the 90kD protein is a proteolysed form of the enzyme whilst the 225kD form could be a dimer. The 60kD CaM-BP was recognised by a polyclonal antibody specific for the

α-subunit of calcineurin whilst the 82kD protein reacted with a polyclonal antibody to chiken gizzard caldesmon (Wallace, Tallant & McManus 1987).

Six CaM-BP's have been identified in the sarcoplasmic reticulum of skeletal muscle. These had molecular weights of 148, 125, 60, 41, 33, and 23kD. It is thought that the 148 and 125kD proteins correspond to the α and β subunits of phosphorylase kinase (Vale 1988).

When the CaM-BP's of rat sperm flagella were analysed by ^{125}I-calmodulin nitrocellulose overlay, only one major protein with a molecular weight of 67kD was identified (Wascoe, Kincaid & Orr 1989). This was found to be calcium dependent as the addition of EGTA inhibited the binding of ^{125}I-calmodulin. Occasionally other lower molecular weight proteins of ≈31, 21 and 15kD were also identified, but these were believed to be proteolytic products of the 67kD protein. To further verify that there was only one CaM-BP the flagella's proteins were cross linked with ^{125}I-calmodulin using dimethyl suberimidate before gel analysis. The result was that two CaM-BPs were identified, with molecular weights of 200kD and 92kD, allowing for the presence of calmodulin (17kD), it was speculated that the 92kD protein was the 62kD protein observed in the primary study. It was also shown that the 67kD protein was not the calcium/calmodulin phosphodiesterase or calcineurin (Wascoe, Kincaid & Orr 1989).

The CaM-BP's of Paramecium cilia have also been identified using both the gel and the nitrocellulose overlay technique with ^{125}I-calmodulin (Evans & Nelson 1989). The most prominently labelled proteins were found to have molecular weights of 126, 96, 63 and 36kD although there was a group of 5 proteins, which formed faint bands, with molecular weights of between 70 and 110kD, a doublet of 45-48kD and a 35kD protein. The 110 and 96kD CaM-BP bound calmodulin both in the presence and absence of calcium, in fact increasing concentrations of calcium above the micromolar range actually inhibited binding of these two proteins to calmodulin. Furthermore, it was found that there were two 110kD CaM-BP's, one that bound calmodulin in a calcium dependent manner and the other that bound in a calcium independent manner. It was also found that the CaM-BP's with molecular weights of 63, 126 & 70-100kD always co-sedimented with tubulin and dynein before and after membrane extraction whilst the 95 and 110kD proteins were always found with the axonemal proteins.

The CaM-BP's of sea urchin eggs have been investigated at two stages of development: the two-cell stage, and the early gastrula stage (Kremier et al. 1992). In both stages of development CaM-BP's with the following molecular weights were observed 200, 150, 50, 43, 41, 35, 34, 21, 18 and 15.5kD. In the early gastrula stage one additional CaM-BP was identified of 70kD whilst in the two-cell stage four additional proteins of 48, 47, 33 and 32kD were found (Kremier et al. 1992). A 50kD CaM-BP has previously been isolated from sea urchin eggs however its function is unknown (Iwasa & Mohri 1983). Also other workers have found CaM-BP's of 55kD and 17kD that have been identified as a calcium/calmodulin dependent phosphoprotein phosphatase (Iswa & Ishuiguro 1986).

The various methodologies used to identify and isolate the CaM-BP's have been criticised. Some workers claim that endogenous calmodulin should be removed before performing calmodulin affinity chromatography (Asselin et al. 1989). However, in practice, removal of endogenous calmodulin is not absolutely necessary. Whilst endogenous calmodulin will inevitably bind to some of the calmodulin binding proteins present within the tissue under investigation there is unlikely to be sufficient calmodulin present to bind to all the calmodulin binding proteins. Consequently there should always be a representative sample of calmodulin binding proteins free to interact with the calmodulin-affinity column. However, when a tissue is known to have an exceptionally high level of calmodulin, e.g. bovine brain or rat testis, then better results might be obtained by removing endogenous calmodulin. There is also the potential problem of proteolysis of the immobilized ligand, or of the bound enzyme or protein. Also, the affinity column may possess non-specific or ion-exchange properties (Asselin et al. 1988). However these factors apply to most chromatographic columns, all of which can be monitored and controlled by appropriate controls and thorough investigation of the optimum conditions for performing the chromatography.

The use of chemically modified calmodulin such as ^{125}I-calmodulin and alkaline phosphatase conjugated calmodulin is also criticised because it involves chemical modifications to calmodulin, which may interfere with its capacity to interact with its target proteins specifically (Asselin et al. 1988). So that despite providing a sensitive procedure for labelling calmodulin binding proteins, either by cross linking or by the gel overlay procedures, extensive characterization of the labelled calmodulin must first be performed.

One solution to these problems has been proposed, which involves using a biosynthetically labelled ^{35}S-calmodulin, which can be produced *in vivo* in E.coli (Asselin et al. 1988). E.coli do not produce endogenous calmodulin and so serve as a useful vehicle for expression of calmodulin mutants. The biosynthetically labelled ^{35}S-calmodulin can then be used in both gel and nitrocellulose overlay procedures.

The CaM-BP's from aortic myocytes have been investigated using the biosynthetically labelled calmodulin but only one CaM-BP was found with a molecular weight of 137kD, which was identified as the myosin light chain kinase (Asselin et al. 1988).

Another problem with either of the overlay techniques, be it of a direct gel overlay of the SDS-PAGE or of a western blot of the gel, is that they both take several days to obtain results. In the case of the gel overlays, it takes 8 days whilst for the western overlays it takes 2 days. In contrast, the use of phenyl Sepharose to isolate calmodulin binding proteins can be performed within one day, with gel analysis overnight.

There is another important fact to consider when comparing not only the techniques used to isolate CaM-BP's but also the reported CaM-BP's. In studies where the calmodulin binding proteins are isolated on a calmodulin agarose column, and then analysed by SDS-PAGE the resulting gel shows both monomeric CaM-BP'S and all the subunits that compose multimeric CaM-BP's that have been dissociated by the denaturing conditions of the gel. In studies where the CaM-BP's are identified by labelling an SDS-PAGE of total protein, by either the gel or the nitrocellulose overlay technique, only the calmodulin binding subunits and monomeric CaM-BP's will be identified. Having considered the various CaM-BP's isolated by other workers, together with the merits of the various methods to identify the CaM-BP's it is now possible to discuss the possible identities of the CaM-BP's isolated in this study.

One of the cestode CaM-BP's of 205kD was found to interact with calmodulin in a calcium dependent and independent manner. Three tissues studied have been shown to have a protein with a similar molecular weight of 200kD: in the nuclear material of yeast, in the nuclei of liver cells and in sea urchin eggs (Bachs et al. 1990; Kremier et al. 1992; Hiragi et al. 1993). However, no reported 200kD protein binds calmodulin in both the presence and absence of calcium.

In all samples of proteins studied proteins with molecular weights between 116kD and 205kD have been found. The protein that was isolated in this project with a molecular weight of between 116 and 205kD was only isolated in the presence of triton. Therefore, this protein is probably membrane bound and could be the calcium ATPase, which has a molecular weight of 130-150kD. Occasionally, another protein was found with a molecular weight between 116kD and 205kD. This could be any one of the following target proteins: *h*-caldesmon (120 or 150kD), calcium/calmodulin protein kinase III (140kD), myosin light chain kinase (128-145kD), adenylate cyclase (135-150kD) or the phosphorylase kinase (β-subunit =128kD and α-subunit=145kD).

During these studies a protein of 116kD was also isolated but a comparable CaM-BP does not appear to have been isolated in other tissue although there are several reports concerning a 110kD protein that is calcium dependent. There is a known CaM-BP that has a β-subunit with a molecular weight of 110kD, which is adducin. It is possible that both the 110kD and the 116kD protein isolated here, are in fact the β-subunit of adducin.

Similarly there are no specific reports on a protein with a molecular weight of 97kD. Although, a membrane bound CaM-BP with a molecular weight of 94kD was identified in rat liver nuclei and a CaM-BP with a molecular weight of 96kD was found in paramecium cilia (Evans & Nelson 1989; Bachs et al. 1990). These proteins could be the α-subunit of adducin, which has a molecular weight of 97kD.

During this study three proteins were isolated with molecular weights between 66 and 97kD. Several other tissues have also been found to contain CaM-BP's with similar molecular weights. There are three possible identities of these proteins: phosphofructokinase (80kD), myosin light chain kinase (77kD) and finally *l*-caldesmon (70-80kD).

A CaM-BP with a molecular weight of 66kD was found in both the cestode and the rat testes. This is particularly interesting because when the rat testes CaM-BP's were isolated, this 66kD protein was the only CaM-BP. Furthermore, a previous study of rat sperm flagella also only found one CaM-BP with the same molecular weight (Wascoe, Kincaid & Orr 1989). Several other tissues have also been shown to contain a protein with a molecular weight of between 62 to 68kD. A 66kD protein has been isolated from the nucleus of Saccharomyces cerevisae, the nuclei of liver cells, the replitase multienzyme complex and the rod outer segments of frogs (Serratosa et al. 1983; Nagao, Yamazaki & Bitensky 1987; Liu et al. 1990; Bachs et al. 1990; Subramanyam et al. 1990; Hiraga et al. 1993). The protein found in rod-outer-segments of frogs is different from all the other reported 67kD CaM-BP's because it bound calmodulin both in the presence and absence of calcium. Consequently it is very likely that this protein is not the same as the one found in other tissues. However the identity of this 67kD protein has not yet been established.

Generally two proteins were found with molecular weights between 45 and 66kD. Similarly many other workers also report finding proteins within this range. There are several possible identities of these proteins. They could be the calcium/calmodulin dependent phosphodiesterase (61-63kD), calcineurin (α-subunit =60kD), inositol 1,4,5, triphosphate kinase (subunit = 53kD), the calcium/calmodulin dependent protein kinase II (57-60kD for the β and β'-subunit), tubulin (50 and 55kD) and finally the τau protein (55-62kD).

A very strong band of protein was always found with a molecular weight of 45kD, in these studies, both in the presence and absence of calcium. Most other workers also report a protein of between 42-48kD. It is likely that this is the γ-subunit of phosphorylase kinase.

A protein with a molecular weight of 36kD and another with a molecular weight of among 36 and 45kD was also found in this study. These two proteins are very probably the calmodulin protein kinase I, which has two subunits with molecular weights of 37 and 39kD, the 36kD protein being the 37kD subunit and the other protein being the 39kD subunit. Both of these subunits bind calmodulin in a calcium dependent manner. Most other tissues studied also contained calcium dependent CaM-BP's with molecular weights ranging from 32-39kD.

Only one protein with a molecular weight of 24kD was found during this study. However, in most other tissues investigated a wide range of proteins between 24 and 30kD have been reported. In this study other proteins were sporadically found when analysing different preparations of CaM-BP's but it was thought that these were likely to be proteolytic products of larger proteins. However, the 24kD CaM-BP was found repeatedly when detergent was included in the isolation material. Therefore, it is possible that this is neuromodulin, which has a molecular weight of 24kD.

Finally proteins were observed which were smaller or equal to 20kD. There are four known contenders for these proteins that include the δ-subunit of phosphorylase kinase, which is in fact calmodulin (17kD), the β-subunit of calcineurin (19kD), histone 2B (19kD) and finally neurogranin (15-19kD).

It can be seen that most of the isolated CaM-BP's from the cestode and from the rat testes appear to correspond with the CaM-BP's isolated from other tissues. Furthermore, it would seem that most of these CaM-BP's have molecular weights in keeping with known CaM-BP's. Even more interesting is the fact that the possible identities of these CaM-BP's appears to correlate with the immunocytochemical localization of calmodulin within the cestode tissue

4.3 Immunocytochemistry

The investigation into the distribution of calmodulin, throughout the tapeworm, revealed that calmodulin was located to a wide range of tissue components. This reflects the wide range of cellular processes that are known to be regulated by calmodulin in higher eukaryotes.

Calmodulin was found associated with two structures that were composed of microtubules, the flame cell and the spermatazoan. Flame cells are the terminal structures of the protonephridial system (section 1.2.1.1) that are found throughout the strobila and scolex. They serve primarily to remove metabolic wastes from the body fluids, although they appear to have some ability to resorb physiologically valuable substances from the filtrate, in common with true kidneys (Lumsden & Specian 1980). Flame cells are composed of 50 or more cilia with each cilium containing an axoneme, which consists of a circle of nine doublet peripheral microtubules and a pair of single microtubules in the centre. The axoneme stems from a basal body composed of nine triplet microtubules and a short rodlet. Within the flame cell there are secondary microvilliform structures that have been called 'leptotriches' (Kummel 1964; Lumsden & Specian 1980; Lumsden & Hildreth 1983). Two enzymes have been found associated with the excretory system, a phosphatase and a Mg^{2+}ATPase, both of which could be regulated by calcium/calmodulin. The cestode spermatozoa are slightly more unusual in that they have a central axoneme with a 9+1 arrangement instead of 9+2.

Many aspects of the cytoskeleton have been shown to be regulated by calmodulin. Calcium and calmodulin inhibit polymerization and assembly of microtubules whilst depolymerization is stimulated by calmodulin (Klee & Vanaman 1982). Calmodulin has also been shown to be involved in regulating the action of calcium on the dynein ATPases, which are responsible for the movement of microtubules resulting in ciliary and flagellary motion (Klee & Vanaman 1982; Manalan & Klee 1984; Veigl, Vanaman & Sedwick 1984).

Immunofluorescence studies have also revealed the presence of calmodulin in the basal bodies of cilia from Paramecium, Tetrahymena and the freshwater mussel, Ellipto (Means & Dedman 1980). Furthermore, calmodulin has also been shown to be a constituent of the axonemal component of cilia. Further studies have demonstrated that calmodulin is present in the acrosome of mammalian sperm, and in the mid-piece with the fertilization ridge. As assembly of the axoneme can be controlled at both ends it has been proposed that calmodulin may play a similar role in sperm as it does in the mitotic apparatus during mitosis, as discussed later (Means & Dedman 1980).

It seems plausible that calmodulin may be regulating both microtubule and dynein-like ATPase activities in the cestode. Flame cells may be undergoing a constant regeneration of microtubules, which may require calmodulin to stimulate depolymerization of existing microtubules. Furthermore, as the cilia in the flame cells move to propel fluid throughout the excretory system, and the microtubules in the sperm cells are acting as flagella, it is possible that there are dynein like ATPases present.

In the cytons, myocytons and tegumentary cytons, calmodulin was located to the cytoplasm, nuclear membrane, nucleus, nucleolus and associated with chromatin in the nucleus. Occasionally it was also observed associated with mitochondria and gap junctions between cytons. A number of workers have shown that calmodulin is involved in nuclear processing. Immunofluorescence studies have shown that calmodulin is associated with the mitotic apparatus of mouse fibroblast, 3T3 and PtK2 cells during all stages of mitosis (Means & Dedman 1980; Cheung 1982). The fluorescence observed, was most intense at the spindle poles and projected towards the chromosomes, which gave the appearance of fibres. However, the observed immunofluorescence did not cross over the metaphase plate that separates the two sets of chromosomes. It was suggested that calmodulin might be preferentially associated with the chromosome-to-pole microtubules and consequently might be involved in the assembly and dissassembly of microtubules during the movement of chromosomes from the metaphase plate to the spindle poles. Further experiments with these cells using an electron microscope revealed that calmodulin was very close to the centrioles and to the kinetochores (Means & Dedman 1980). Consequently, it has been proposed that during mitosis, calmodulin functions to depolymerize the microtubules in the polar region so shortening the kinetochore-to-pole microtubules and thereby allows movement of the chromosomes to the poles. Calmodulin has also been located to the smooth endoplasmic reticulum that lies parallel to the mitotic spindle, and to membrane vesicles and mitochondrial membranes. It is thought that calmodulin might be regulating calcium pumps at these sites (Means & Dedman 1980).

It has also been shown that calmodulin is involved in triggering DNA replication (Bachs et al. 1990). For example, reduction or elevation in the expression of the calmodulin gene, and therefore intracellular calmodulin levels, in C127 mouse cells' results in either prolonging or shortening the length of the G1 phase of the cell cycle (Bachs et al. 1990). This increase in calmodulin concentration before DNA synthesis has also been observed in other cell types, for example CHO-K1 cells (Chafouleas et al. 1982; Bachs et al. 1990). Furthermore, calmodulin is required to stimulate DNA synthesis during Planarian regeneration (Martelly et al. 1983). Finally it has also been shown that DNA replication is inhibited by antagonists of calmodulin (Bachs et al. 1990).

An immunocytochemical study, at the light microscope level, revealed that calmodulin formed dense aggregates in the nuclei of quiescent liver cells. However, when nuclei were observed from a hepatectomized liver, calmodulin was found associated with the nuclear membrane and the dense aggregates had diffused (Serratosa et al. 1988). Another immunocytochemical study into a CaM-BP protein, p62, was also performed on hepatectomized liver sections (Bachs et al. 1990). This revealed that p62 was located primarily to the nuclear envelope. However, some label was also associated with condensed aggregates of chromatin. This pattern, of labelling, is very similar to the pattern observed for calmodulin in hepatectomized liver cells.

Another immunocytochemical study using electron microscopy revealed that calmodulin was associated with a variety of intracellular membranes that included nuclei, the Golgi apparatus, endoplasmic reticulum, plasma membrane, mitochondria and post-synaptic membranes (Lin et al. 1980).

The muscle fibres were the most highly labelled region throughout the tapeworm. Cestodes only contain smooth muscle so that this finding is particularly significant. Smooth muscle contraction in eukaryotes is controlled by calmodulin and is generally responsible for slow, long lasting and involuntary contractions. Calcium/calmodulin interacts with myosin light chain kinase converting it into its active form, which can then catalyse the transfer of P_i from ATP to the regulatory light chain of myosin resulting in myosin contraction. Relaxation is achieved by a phosphatase that dephosphorylates the myosin light chains. In cestodes the contractile myofibrils that compose the smooth muscle are served by myocytons that contain the nucleus and glycogen stores together with lipid deposits (section 1.2.1.1). These myocytons are frequently some distance from the contractile myofibrils that they serve, being connected by tendrilled cytoplasmic processes (Lumsden & Bryam III 1967; Lumsden & Hildreth 1983). Immunofluorescent studies of skeletal muscle calmodulin have shown that calmodulin is located to the I-band and the sarcoplasmic reticulum (Cheung 1982).

In the neck region an organelle, containing electron dense granules, was found that was always packed with gold label. It is probable that this organelle is neurosecretory as the scolex and neck region have been shown to have a relative abundance of neuronal tissue (Wilson & Schiller 1969; Webb & Davey 1975; Webb & Davey 1976). Nervous tissue in cestodes lacks a definite capsule or sheath, but forms a fairly extensive network of nerve ganglia and 'fibres' (section 1.2.1.1.). However, the only neurotransmitters identified in H.diminuta are acetylcholinesterase and 5'hydroxytryptamine (Wilson & Schiller 1969; Webb & Davey 1975; Webb & Davey 1976; Specian et al. 1979; Specian & Lumsden 1980). In the tail region larger organelles were found that contained electron lucent granules. It is possible, that these could either be the penetration gland of the oncospheres or it could be another neurosecretory organelle.

There is considerable evidence that calmodulin has a central role in regulating neuronal processes. Much evidence comes from the vast array of target proteins that have been identified in the brain, and some evidence that comes from immunocytochemical studies. One such study showed that calmodulin was located to the post synaptic region of nerve cells. Furthermore, when rat adrenal cortex is injected with corticotrophin (ACTH), which stimulates steroid synthesis, after 11 hour's calmodulin that had previously been located in the cytoplasm was found in the nucleus of these cells, with none in the cytoplasm (Cheung 1982).

The distribution of calmodulin in the rat cerebellum has also been studied using immunocytochemistry. Calmodulin was found to be concentrated in the Purkinje and glial cell bodies, where it was predominantly associated with free and bound ribosome's, the nuclear envelope, coated membranes of smooth and rough endoplasmic reticulum, and mitochondrial and plasma membranes. Furthermore calmodulin was also found in the neuronal dendrites of the Purkinje cells, in which it coated the surface of smooth endoplasmic reticulum small vesicles and the inner and outer mitochondrial membranes (Means & Dedman 1980).

One of calmodulin's target proteins is the multifunctional calcium/calmodulin protein kinase (I, II and III) that phosphorylates proteins at serine and threonine residues. It is involved in the regulation of neurotransmitter synthesis and release, carbohydrate metabolism, nuclear envelope breakdown and neuronal plasticity (Nairn, Hemmings & Greengard 1985; Cohen 1988; MacNicoll, Jefferson & Schulman 1990). It is also the major kinase involved in the co-ordination of cellular responses to hormones and neurotransmitters. Both of which increase the calcium levels by influx through ligand gated channels and by voltage sensitive channels. It also responds by redistributing the calcium from inositol phosphates (IP_3) and caffeine sensitive stores (Schulman 1992). Therefore the observed labelling, in the cestode, of calmodulin in these two organelles, containing electron dense and electron lucent granules, could be to calmodulin associated with the multifunctional protein kinase.

The tegument including both the microthrix and the distal cytoplasm was also frequently labelled. The microthrix layer is constantly being repaired and regenerated and therefore requires large amounts of energy and raw materials (Lumsden & Specian 1980). Consequently, it is probable that a large number of cellular processes and messenger systems are in operation, some or all of which may be regulated by calcium and calmodulin. As the tegument is also the absorptive and excretory surface of the worm it contains a large number of transporters. Both phosphodiesterase and ATPase activities have been found, which are stimulated by calcium and magnesium ions, both of which could therefore be regulated by calmodulin (Gamble & Pappas 1981; Pappas 1981). Furthermore, it is possible that one of the CaM-BP's isolated was a Ca^{2+}/Mg^{2+}ATPase. In the sarcoplasmic reticulum of higher eukaryotes the ATPase provides the energy to accumulate calcium against the electrochemical gradient that exists across the membrane. Generally the Ca^{2+}/Mg^{2+}ATPase is regulated by calmodulin to terminate calcium messengers so that intracellular calcium concentrations return to resting levels. It is possible that, in the cestode, the ATPase found in the tegument controls intracellular calcium levels in response to external conditions, and that the ATPase is controlled by calmodulin.

Calmodulin was frequently found associated in the cytoplasm around internuncial processes, which extend from the tegumentory cytons to the distal cytoplasm crossing through the basal lamella. Cellular material is transported through these processes between the cell body and the distal cytoplasm (Lumsden & Hildreth 1983). The distal cytoplasm contains large numbers of vesicles, granules and mitochondria (Lumsden & Hildreth 1983).

Somewhat surprisingly no calmodulin was found associated with the ovaries. However, calmodulin was found in the oncospheres, within the different envelope layers. Identification of the components of the oncosphere was difficult, partly because of the high magnification being used to visualise the calmodulin and partly because the oncosphere is continually changing throughout its development (Section 1.2.1.1.). Calmodulin has been identified in the oocytes of Chaetopterus, starfish, Xenopus and mouse oocytes (Meijer & Wallace 1985). However when the calmodulin binding proteins have been analysed from these oocytes, only phosphodiesterase and the calmodulin multifunctional protein kinase have been found. Also the levels of calmodulin detected have been extremely low compared to other tissue sources of calmodulin. In Chaetopterus there is only 1.7μg/mg protein, whilst starfish oocytes contain between 130 and 200ng of calmodulin per oocyte. Pleurodeles oocytes contain around 220ng/oocyte and mouse oocytes contain 3μg/mg protein. It is only in Xenopus oocytes that calmodulin appears to have a specific role. If Xenopus oocytes are treated with progesterone, the levels of calmodulin remain constant for approximately 2-2*1/2* hours. Then concomitant with the rupture of the nuclear envelope there is a 30-80% increase in the levels of calmodulin.

Frequently calmodulin was found in the cytoplasm of the cytons. In the myocytons, in particular, there are large stores of glycogen (section 1.2.2.2.). To maintain these stores the cestode must expend considerable energy and resources in carbohydrate metabolism and catabolism. H.diminuta possess glucose-6-phosphate glycogen synthase and a protein kinase and phosphatase that are responsible for the conversion of glucose 6-phosphate between the glucose 6 phosphate dependent and independent forms (Read 1951, Dendinger & Roberts 1977; Moczon 1977a & b). Glycogen phosphorylase, phosphorylase *a* phosphatase and phosphorylase *b* kinase, cAMP dependent protein kinase and 1,4-α glucan branching enzyme have all been shown to be present in H.diminuta (Moczon 1975, 1977a & b). As already stated, in section 1.2.2.2., it is thought that cestodes use the Embden Meyhof pathway for glycolysis as hexokinase, pyruvate kinase, lactate dehydrogenase, phosphoenol pyruvate carboxykinase and malate dehydrogenase have all been found in the cestode (Prescott & Cambell 1965; Bueding & Saz 1968; Burke et al. 1972; Moon et al. 1977; Kommunieki & Roberts 1977). One of the calmodulin binding proteins identified could be phosphorylase kinase, as proteins with molecular weights representing each of its subunits were found (α=145kD, β=128kD, γ=45kD and δ=17kD). Phosphorylase kinase (also called glycogen phosphorylase *b* kinase) controls' glycolysis by phosphorylating serine 14 of glycogen phosphorylase converting it from the inactive *b* form to the active *a* form (section 1.1.1.1.2.). Another CaM-BP that was isolated could have been phosphodiesterase, which converts cAMP to 5'AMP and so controls the levels of cAMP found in cells. cAMP levels are involved in the regulation of glycogen metabolism, as they control the protein kinase regulator that converts protein kinase into its active form, which in turn activates glycogen phosphorylase *b* kinase. See Figure 96. These findings provide further evidence that glycogen metabolism in H.diminuta is very similar to that found in higher eukaryotes. Calmodulin was occasionally found near to gap junctions between cytons. In the past gap junctions in cestodes have been referred to as desmosomes however it has been argued that these structures appear more like "gap' or 'nexus' junctions than desmosomes (Lumsden & Specian 1980). Gap junctions have been observed between two muscle cells, muscle and tegument, and muscle and associated endocrine cells .

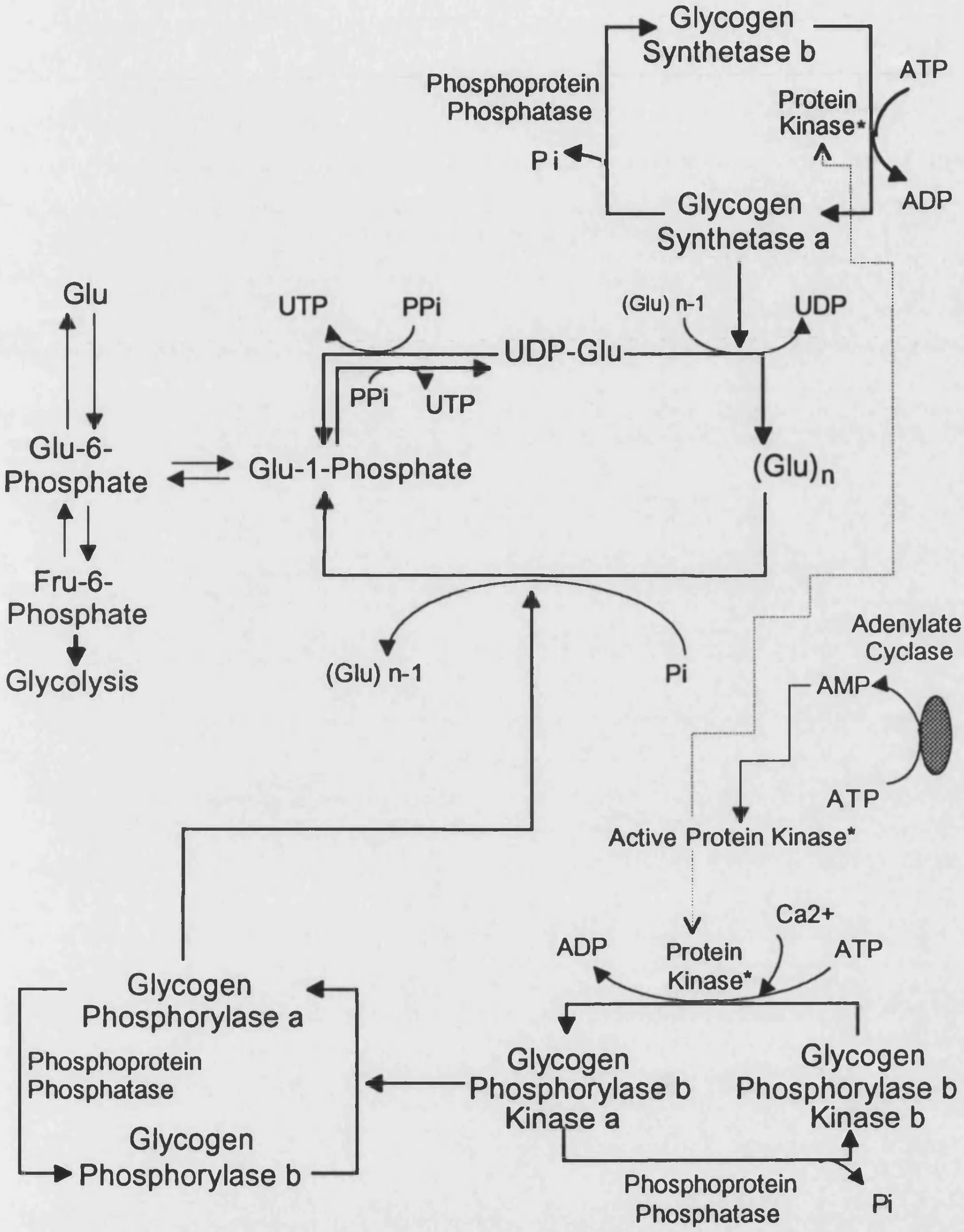


Figure 96 Glycogen Metabolism

Glucose is converted into glucose-1-phosphate that can then be used to synthesise glycogen. Degradation of glycogen is regulated by a cascade of reactions terminating in the stimulation of glycogen phosphorylase. The whole process is regulated by cellular levels of cAMP.

(Lumsden & Specian 1980). If these structures are true gap junctions the labelling of calmodulin near to these structures is particularly interesting. True gap junctions are composed of arrays of cell-to-cell channels that are composed of connexins. Connexins oligomerize to form hemichannels (connexons) which align on adjacent cells to provide channels between cytoplasms through which ions and metabolites up to 1kD can pass (Hennemann et al. 1992).

There are twelve connexin isoforms, but there are three major ones that are widely expressed in tissue. These are connexins Cx26, Cx32 and Cx43. On each connexin there are two intracellular calmodulin binding sites, one of which is on the N-terminus and the other is on the C-terminus (Evans 1994). Furthermore, the calcium/calmodulin dependent protein kinase II is implicated in regulating the opening and closing of these channels (Elvira et al. 1993).

4.4 Conclusions and Future Directions

This project was originally undertaken to discover more about calmodulin within the cestode. Calmodulin had previously been isolated from H.diminuta by Hipkiss (1986) and had been shown to have a molecular weight of 16.9kD by SDS-rod gel electrophoresis. It was also shown that cestode calmodulin would stimulate phosphodiesterase. Consequently, one of the initial goals of this study was to isolate the calmodulin gene and obtain the nucleotide sequence so that comparisons could be made between cestode calmodulin and calmodulin from other organisms. However, insubstantial quantities of DNA were obtained to prepare a genomic library and attempts at cloning and sequencing the calmodulin gene, using the polymerase chain reaction, proved unsuccessful. Whilst attempts at obtaining cDNA were promising, this line of work had to be put on hold due to lack of time and money.

Whilst the molecular biology had to be abandoned during this project, isolation of the gene is still necessary to understand more about calmodulin within the cestode. Ideally formation of a genomic library or cDNA library would prove extremely useful as one could also investigate the genes for the calmodulin binding proteins. Once a clone of the calmodulin gene has been obtained, the nucleotide sequence can be determined and compared with other calmodulin nucleotide sequences to assess the degree of conservation throughout evolution. Moreover, valuable information concerning the regulatory coding sequences used by cestodes could also be ascertained.

Instead, it had been hoped that the amino acid sequence could have been obtained directly from the isolated protein. A new method was devised to isolate calmodulin from the cestode, first using pig thymus to establish the protocol. Four methods were tried all of which used hydrophobic chromatography as a key isolation step. The optimum method for isolating pig thymus calmodulin was established and the same method was tried with cestode, however the initial results were disappointing. It was noticed that 50% of pig thymus protein was soluble compared to 90% found in the cestode. Consequently, all the methods tried with pig thymus were used to isolate calmodulin from the cestode. It was found that the first method, using heat treatment followed by phenyl sepharose chromatography, yielded the most homogeneous preparation of cestode calmodulin. When this was analysed by SDS-PAGE it migrated with a molecular weight of around 17kD and showed a migratory shift, characteristic of calcium binding proteins, in the presence and absence of calcium.

Unfortunately, insufficient calmodulin was obtained to determine the amino acid sequence. However, there are many companies that now offer a service for sequencing very small quantities of proteins. The protein of interest has to be separated on a polyacrylamide gel and then transferred to a special nitrocellulose membrane on which it has been renatured. The membrane together with the protein is then dispatched and the company performs sequence analysis. Unfortunately this was too costly to pursue during this project but it could be used in the future.

Furthermore a study to determine if cestode calmodulin has undergone any post-translational modifications, i.e. whether the N-terminus is blocked by acylation and whether the lysine at position 115 is trimethylated, also needs to be performed. The effect of various proteolytic enzymes also needs to be investigated.

The calmodulin obtained in this study was tested for its biological activity using the phosphodiesterase assay system. Whilst this is a very traditional assay to use, it is somewhat unreliable as it was found that the enzyme itself was unstable. If this assay system is to be used in future studies then the phosphodiesterase must be prepared immediately before the experiment. Alternatively, it is possible to use any one of the calmodulin target proteins to assay for the biological activity of calmodulin. For example the Mg^{2+}ATPase of erythrocytes is calmodulin activated and has been used to test calmodulin from trypanosomes (Ruben, Egwuagu & Patton 1983). In this experiment human erythrocytes are washed in isotonic buffer, lysed and the membranes isolated. The activity of the Ca^{2+}/Mg^{2+}ATPase is established at 37°C in a medium containing 0.1mM oubain, 40mM NaCl, 3mM $MgCl_2$, 7.5mM KCl, 20mM glyclglycine pH7.1 Then reactions are set up with 0.5mM EGTA, 150μM Ca^{2+} or 1μg/ml calmodulin. The reaction is started by the addition of 2mM ATP, and stopped by the addition of 5% TCA (final concentration) and samples are taken throughout the procedure. The phosphate released is then measured by the method of Fiske and Subbarow (1925). Measurement of the Ca^{2+}ATPase activity has also been used to study the effects of antagonists on calmodulin stimulation of the calcium pump (Vincenzi 1982).

The phosphorylation of caldesmon by the smooth muscle calmodulin dependent protein kinase II has also been used to test the biological activity of calmodulin (Ikebe & Reardon 1990). In this experiment, 0.2mg/ml caldesmon is mixed with 10μg/ml of smooth muscle protein kinase II and incubated at 25°C in a solution containing 5mM $MgCl_2$, 0.2mM $CaCl_2$, 5mg/ml calmodulin and 30mM Tris-HCl pH 7.5. The reaction is started by the addition of 0.2mM γ-^{32}P-ATP (25000cpm/mol). Aliquots are removed at intervals and the reaction stopped by the addition of 5% TCA and 1% pyrophosphate. The radioactivity can then be measured by Cherenkov counting.

An investigation into calmodulin within the cestode would not be complete without investigating the target proteins with which it interacts. Isolation of the calmodulin binding proteins, with calmodulin agarose, is a preliminary step in identifying these targets. It proved a highly successful method to use and several calmodulin binding proteins were found that had comparable molecular weights with known CaM-BP's. These possible identities included α-spectrin, caldesmon, protein kinase I and II, Ca^{2+} ATPase, myosin light chain kinase, phosphorylase kinase, adenylate cyclase, adducin *a* and *b* subunits, phosphodiesterase, calcineurin, tubulin, tau protein, neuromodulin, neurogranin and histone 2b. The presence of such a large number of calmodulin binding proteins suggests that calmodulin and by default calcium must play an important role in the metabolic pathways in cestodes.

During this study the precise molecular weights for the calmodulin binding proteins were not determined. This is because the proteins were analysed on a 12% gel, which does not satisfactorily resolve high molecular weight proteins, because they cannot enter the gel matrix, or very small molecular weight proteins, because they run at the dye front and could be lost. If the proteins were analysed on a 7% gel then the high molecular weight proteins would be separated and their molecular weights could be determined. Likewise, if the calmodulin binding proteins were analysed on a 15-20% gel then the molecular weights of the low molecular weight proteins could also be determined. If possible, the gels should also be scanned, so that the relative amount of protein in each of the constituent bands can be calculated. Furthermore, the molecular weights of each of the calmodulin binding proteins could be determined by performing gel filtration on the fractions obtained from the calmodulin agarose column. This would also enable each protein to be isolated in its native state.

The cellular distribution of the calmodulin binding proteins could also be investigated by performing subcellular fractionation of the tapeworm tissue before calmodulin affinity chromatography. Analysis of these proteins by SDS-PAGE would reveal the calmodulin binding proteins found within each of the cellular compartments, e.g. nuclear, mitochondrial, membrane, etc.

Obviously, the calmodulin binding proteins also need to be further identified. An overlay technique could be performed to determine which of the protein bands found in the SDS-gel, be it a subunit or a monomeric protein, actually binds calmodulin. Similarly the calmodulin binding proteins could be analysed on a native gel then they could be labelled with calmodulin using one of the overlay procedures. Furthermore, the proteins could even be cross-linked with calmodulin before gel electrophoresis. Both of these studies would verify that the proteins isolated from the calmodulin affinity column were true calmodulin binding proteins. Antibodies have been produced to a wide range of calmodulin binding proteins. Consequently these could be used in both ELISA's and western blots to identify the isolated calmodulin binding proteins. Furthermore, if the amino acid sequence was determined, not only would the identity of the calmodulin binding proteins be revealed, but also information concerning the conservation of proteins throughout evolution.

Once identified it would be possible to prepare oligonucleotide probes, which could be used to isolate the genes encoding the CaM-BP's from a genomic or cDNA library of H.diminuta. This additional information would tell us more about the role of calmodulin and its target proteins within the cestode, and the metabolic processes that are involved. It would also give new insights into metabolic pathways that could be targeted for treatment by antihelminthic drugs.

The calmodulin binding proteins that were isolated tied in very well with the results obtained in the immunocytochemical study of calmodulin. This study showed that calmodulin was localized to structures that have previously been shown to contain calmodulin in higher eukaryotes, be it by immunocytochemistry or by subcellular localizations. Calmodulin was localized to muscle fibres and one of the calmodulin binding proteins could have been myosin light chain kinase, which is known to regulate smooth muscle contraction. The presence of another calmodulin binding protein, phosphorylase kinase, reflects the large stores of glycogen in the cestode and probably, partly accounts for the distribution of calmodulin throughout the cytoplasm. A calmodulin binding protein was found with a molecular weight comparable to the calcium ATPase. The localization of calmodulin to the tegument could possibly be to a Ca^{2+}/Mg^{2+}ATPase that has previously been reported by other workers (Hipkiss 1986). The finding of a potential ATPase also fits in with the localization of calmodulin to flame cells and spermatazoan, both of which are thought to contain an ATPase. Furthermore, cestodes contain a 67kD calmodulin binding protein that has been found in the spermatozoa of other organisms, and is the sole calmodulin binding protein isolated from rat sperm flagella. This was also the only calmodulin binding protein recovered from rat testes in this study.

The possible presence of phosphodiesterase, calcium/calmodulin protein kinase and calcineurin, a protein phosphatase corresponds to the calmodulin that was localized to the cytoplasm.

These two aspects of this investigation, the immunolocalization of calmodulin and the isolation of calmodulin binding proteins, have revealed that calmodulin obviously plays a central role in regulating cellular processes in response to changes in cellular calcium levels in the cestode. However, the immunocytochemistry could be improved upon. A different resin such as K4M could be used which would enable better infiltration of the cestode material, so more extensive studies could be performed on the lower regions of the cestode tissue. K4M is a Lowicryl resin that other workers have used for immunocytochemical work in parasites. The Lowicryl resins were formulated to provide embedding media that possessed low viscosity and maximised the preservation of the molecular and antigenic structure of the cell. All Lowicryl resins have a low viscosity, even at low temperatures, and are polymerized by UV light at low temperature (Monaghan, Robertson & Beesley 1993). K4M can be used for low temperature embedding with the progressively lowering of the temperature to -40°C during dehydration in alcohol, and renders tissue proteins insoluble. Consequently, the proteins are unlikely to leach from the tissue and molecular movement is hindered. The main drawback to this method is the requirement of a freezer containing a UV light source for the polymerization of the resin at -40°C.

It would be interesting to try a quantitative study of the immunocytochemical localization of calmodulin. Also, the presence of both the calmodulin binding proteins and calmodulin could be further investigated by performing immunocytochemistry, at the electron microscopy level, using dual labelling. Here the tissue could be incubated with calmodulin and an antibody to one of the calmodulin binding proteins. Furthermore, this technique could be expanded to incorporate studies on the effects of antihelminthics on the distribution of calmodulin and/or its target proteins.

It would also be of interest to perform the immunocytochemical localization of calmodulin and CaM-BP's on both the cysticercoids and isolated oncospheres as only adult tapeworms were used in these studies. Similarly, isolating the CaM-BP's from each stage of the life cycle would provide insights into changes in metabolic pathways .

To conclude then this study has provided further evidence that calmodulin is involved in cellular processes in the cestode. Furthermore the presence of a substantial number of calmodulin binding proteins suggests that calmodulin has a central role in cestode metabolism, which compares with that found in higher eukaryotes. However, the precise nature of these metabolic pathways needs further elucidation as more questions have been raised in this study than have been answered. Only further investigation will reveal the full extent of calcium and calmodulin regulation in cellular events in the cestode. If these studies are performed then the increased knowledge of cestode metabolism will enable more efficient anti-helminthics to be developed that will relieve the suffering of both humans and animals.

APPENDIX 1 DEFINITION OF UNITS

3'5'-cyclic nucleotide Phosphodiesterase

One unit will hydrolyze 1µM 3'5'-cAMP to 5'-AMP per minute at pH7.5 at 30°C.

Calmodulin (Sigma)

One unit stimulates 0.016 activated units of 3'5'-cyclic nucleotide Phosphodiesterase to 50% of the maximum activity when saturated with activator in the presence of 0.01mM Ca^{2+} at pH7.5, at 30°C.

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PRESENTATIONS AND PUBLICATIONS

1. Poster: "The calmodulin gene and its role in the cestodes". South West Region Biochemical Society Predoctoral meeting at Swansea 1991.

2. Oral: "The calmodulin gene and its role in cestodes". Departmental seminar at Bath university, 1991.

3. Paper: "Molecular biology of the eucestoda". **Biochemical Society Transactions 20** (1992) p151s; and presented as a poster at 641st Biochemical Society Meeting, 1992 at Royal Holloway and Bedford New College, University Of London.

4. Oral: "Calmodulin and its isolation". Departmental seminar at Bath university 1992.

5. Poster: "Calmodulin from the eucestode Hymenolepis diminuta and its use as a probe for calmodulin binding proteins" at British Society for Parasitology Spring meeting at York 6-8th. April 1992.

6. Paper: "Calmodulin from the eucestoda and its use as a probe for calmodulin binding proteins" **Biochemical Society Transaction** 22 (1992) p300s and presented as a poster at 642nd Biochemical Society Meeting, 1992 at University of Southampton.

7. Poster: "Calmodulins role in the regulation of protein function from the eucestode, Hymenolepis diminuta." at Federation Of European Biochemical Societies 21st Annual Meeting, Trinity College, Dublin, Ireland. Abstract No. Tu101.

8. Poster: "Host-cestode calmodulin-binding protein studies" at 8th. International Symposium On Calcium-binding Proteins And Calcium Function In Health And Disease 23-27th. August 1992 Davos, Switzerland.

9. Oral: "Calmodulin: its distribution in the eucestoda, H.diminuta", Departmental seminar Bath university 1993.

10. Paper: "Immunohistochemistry of calmodulin in the eucestoda, H.diminuta", and Poster: "Calmodulin and calmodulin binding proteins in the eucestoda, H.diminuta." British Society for Parasitology Spring meeting Leeds 4th.-6th. April 1993.

11. Paper: "The distribution of calmodulin/calmodulin binding proteins in the rat tapeworm, H.diminuta." Accepted for publication by Comparative Biochemistry & Physiology.

12. Poster: "The localization of calmodulin/calmodulin binding proteins in H.diminuta." British Society For Parasitology Spring meeting, Bath 6-8th. April 1994.